University of Alberta

CHARACTERIZATION OF AN INDIRECT *BLDA* TARGET FROM *STREPTOMYCES COELICOLOR*

by

JANICE LORRAINE STRAP ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of DOCTOR OF PHILOSOPHY

Department of Biological Sciences

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ABSTRACT

Streptomycetes undergo both biochemical (antibiotic production) and morphological differentiation as part of their complex life cycle. These organisms are of great interest due to their ability to produce an extensive array of chemically diverse, commercially important secondary metabolites (which includes most of the antibiotics currently used in human and veterinary medicine). The *bld* (for bald) mutants of *Streptomyces coelicolor* are unable to complete the first stage of sporulation which involves the transition from a vegetative, substrate mycelium, non-antibiotic-producing mode of growth to an aerial mycelium, antibiotic-producing state.

The best characterized *bld* gene is *bldA* which encodes a leucyl tRNA that recognizes the rare UUA codon in the high G+C (70-74%) *Streptomyces* mRNA. This TTA codon for leucine is confined to a small number of *Streptomyces* genes that are predominantly involved in the regulation of morphogenesis and secondary metabolism.

Manipulation of antibiotic production relies on understanding the nature of the regulatory components involved in the induction of genes responsible for antibiotic biosynthesis. In an endeavor to advance current knowledge of the regulatory role that *bldA* plays in the process of differentiation, attempts were made to identify targets of *bldA* in *Streptomyces coelicolor*. *bldA* targets are TTA-containing genes that require the *bldA*-encoded tRNA for translation of their mRNAs. Since *bldA* acts at the level of translation, protein profiles from *bldA*$^+$ and *bldA*$^-$ strains were compared. To narrow the search for the small number of expected *bldA* targets, *in vitro* protein phosphorylation was used. As a result of this analysis, a 32 kDa protein that exhibited a higher level of phosphorylation in surface culture cell-free extracts from a *bldA*$^-$ strain compared to a

bldA$^+$ strain, was identified. Although the DNA sequence of the gene encoding this protein product was devoid of TTA codons, the apparent *bldA*-dependent phosphorylation status of this protein suggests it is an indirect *bldA*-target. Indirect *bldA* targets can be generally defined as proteins that do not possess TTA codon(s) but are regulated by a TTA-containing regulator. The use of a reverse genetics approach to clone the gene for this target identified a sequence that would encode the alpha (α) subunit of the succinyl-CoA synthetase (SCS) of the tricarboxylic acid (TCA) cycle. Western analysis with anti-phosphotyrosine antibodies indicated that the 32 kDa protein possessed phosphotyrosine. Furthermore, phosphoamino acid analysis of this protein confirmed the presence of phosphotyrosine and also demonstrated the presence of phosphoserine and phosphothreonine. Since attempts to construct a null mutation of the gene failed, the role played by this TCA cycle enzyme in the regulation of antibiotic production and morphological differentiation is not yet clear.

Nothing in the world can take the place of persistence. Talent will not; nothing is more common than unsuccessful men with talent. Genius will not; unrewarded genius is almost a proverb. Education will not; the world is full of educated derelicts. Persistence and determination alone are omnipotent.

-- Calvin Coolidge --

One should guard against preaching to people success in the customary form as the main aim in life. The most important motive for work in school and in life is pleasure in work, pleasure in its result and the knowledge of the value of the result to the community.

-- Albert Einstein --

An education isn't how much you have committed to memory, or even how much you know. It's being able to differentiate between what you know and what you don't.

-- Anatole France --

Success is not measured by the position one has reached in life, rather by the obstacles overcome while trying to succeed.

-- Booker T. Washington --

Difficulties increase the nearer we approach our goal.

-- Goethe --

Nil tam difficile est quin quaerendo investigari possiet.

-- Terence --

Potius sero quam numquam.

-- Titus Livius --

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LIST OF ABBREVIATIONS

A	Adenosine
amp	Ampicillin resistance gene
AMV	Avian Myeloblastosis Virus
apr	Apramycin resistance gene
apr^{R}	Apramycin resistant phenotype
apr^{S}	Apramycin sensitive phenotype
APS	Ammonium persulfate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine Serum Albumin
C	Cytidine
CAPS	3-[cyclohexylamino]-1-propanesulfonic acid
CFU	Colony Forming Units
CHAPS	[3-[(3-Cholamidopropyl)dimethylammonio 1-propane-sulfonate]]
Ci	Curie
cpm	Counts per minute
C23O	Catechol-2,3-dioxygenase
DEPC	Diethyl pyrocarbonate
ddNTP	Dideoxynucleside triphosphate
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ermE	Erythromycin resistance gene (or promoter)
EtBr	Ethidium Bromide
G	Guanosine
HEPES	*N*-2-hydroxyethylpiperazine-*N*'2-ethane sulfonic acid
HRP	Horse Radish Peroxidase
IEF	Isoelectric Focusing

IPTG	Isopropyl β-D-thiogalactopyranoside
kb	kilobase
λ	Lambda Bacteriophage
LB	Luria-Bertani medium
LSC	Liquid Scintillation Counting
MCS	Multiple cloning site
MMGTM	Minimal Medium supplemented with glucose and Tiger Milk
MW	Molecular weight
NTP	Nucleoside triphosphate
ORF	Open reading frame
P Buffer	Protoplast buffer
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
PFU	Plaque Forming Units
PMSF	Phenylmethylsulfonyl Fluoride
PVDF	Polyvinylidene difluoride
PY	Phospho-Tyrosine
R2YE	Sucrose Yeast Extract Medium
rpm	Revolutions per minute
SCS	Succinyl-CoA Synthetase
SDS	Sodium dodecyl sulfate
SNA	Soft Nutrient Agar
SPSO	Single plaque soak out
SSC	Standard Saline-Citrate
T	Thymidine
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
T_d	Dissociation temperature
TBS	Tris Buffered Saline
TE	Tris-EDTA Buffer

TEMED	*N,N,N',N'*-tetramethylethylenediamine
TES	N-tris [Hydroxymethyl] methyl-2-aminoethane sulfonic acid
TLC	Thin layer chromatography
T_m	Melting temperature of DNA hybrids
Tris	Tris(hydroxymethyl)aminomethane
TSB	Trypticase Soy Broth
tsr	Thiostrepton resistance gene
UV	Ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
xylE	Catechol dioxygenase gene
YEME	Yeast Extract-Malt Extract Medium

I INTRODUCTION

I.1 Overview of Streptomycetes

Members of the genus *Streptomyces* belong to the order *Actinomycetales* and are filamentous, Gram positive, soil organisms of industrial significance. These bacteria undergo both chemical (antibiotic production) and morphological differentiation as part of their complex life cycle (Chater, 1993). The streptomycetes are of great interest due to their ability to produce an extensive array of chemically diverse secondary metabolites which serve as commercially important compounds including medically important anti-microbial, anti-parasitic, anti-tumor and anti-cancer agents to agriculturally important herbicides (Bibb, 1996b). Secondary metabolites are usually produced in a growth-phase dependent manner (Demain and Fang, 1995). The onset of morphological differentiation, which is characterized by the development of aerial hyphae and spores, is often coincident with the onset of antibiotic production suggesting that these two processes are activated coordinately (Champness and Chater, 1994). The multicellular mode of growth of these organisms requires the interplay of many levels of regulation and signalling mechanisms. At present, the intricacies of these mechanisms remain to be elucidated.

I.1.1 *Streptomyces coelicolor* is Amenable to Genetic Characterization

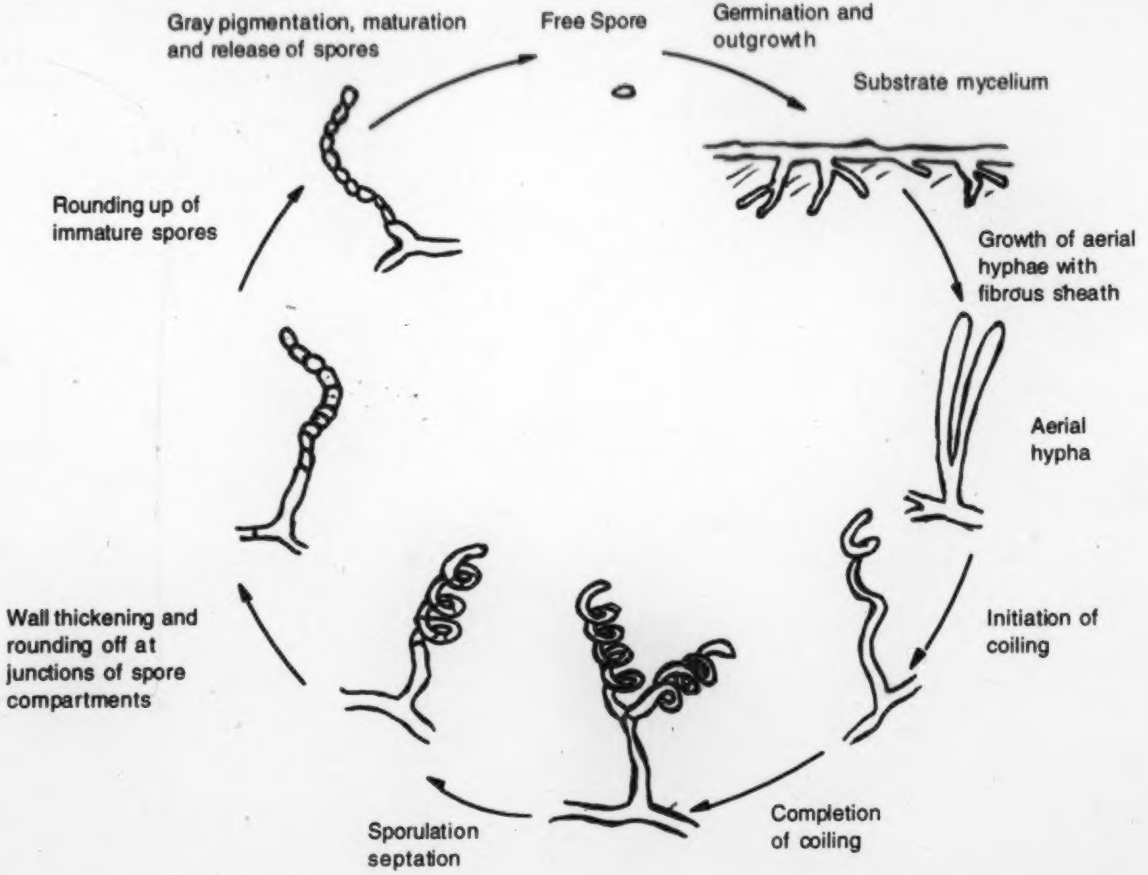
S. coelicolor A3(2) is particularly useful for the study of the regulation of antibiotic production since it has been genetically well characterized (Hopwood, 1988), and it produces four distinct antibiotics which permit the analysis of pathway-specific and pleiotropic regulation of antibiotic production (Champness, 2000; Champness and

Chater, 1994). In liquid culture, *Streptomyces coelicolor* is unable to undergo morphological differentiation but is able to produce antibiotics. The knowledge gained from the study of the regulation of antibiotic biosynthetic pathways along with studies of the regulation of morphological differentiation will allow development of novel approaches for strain improvement for the production of compounds with industrial applications (Baltz and Hosted, 1996; Chater, 1990) as well as enable the creation of novel antimicrobial agents. Since secondary metabolism is responsive to developmental as well as environmental signals its regulation is of fundamental scientific interest.

I.1.2 The Life Cycle of *Streptomyces*

On a solid surface, a single, uninucleate spore in the presence of favorable environmental conditions can germinate and give rise to a complex vegetative mycelial mat that grows on and into the growth substrate. In response to as yet undetermined signals, presumed to involve nutrient depletion, the process of differentiation is initiated and leads to the formation of multi-genomic aerial hyphae that protrude from the surface of the substrate mycelium into the air to bestow a characteristic fuzzy appearance to the developing colony (Figure I.1.2.1). Lysis of the substrate mycelium accompanies the formation of aerial mycelium which is believed to provide a nutritional source for later developmental processes (Mendez *et al.*, 1985; Miguelez *et al.*, 1994; Wildermuth, 1970) since the aerial mycelium has limited access to other nutritional sources (Chater, 1984). The mechanism by which nutrients are transported throughout the aerial mycelium is still not understood. The aerial hyphae undergo relatively synchronous septation and are

Figure I.1.2.1. Life cycle of *Streptomyces coelicolor*. The development of *Streptomyces* from the unigenomic spore through substrate and aerial mycelia is shown. The *bld* genes are involved in the production of aerial hyphae while the *whi* genes are involved in the maturation of aerial hyphae into chains of pigmented spores (see text for details). Adapted from Chater and Merrick (1979).


Free Spore
Germination and outgrowth
Substrate mycelium
Growth of aerial hyphae with fibrous sheath
Aerial hypha
Initiation of coiling
Completion of coiling
Sporulation septation
Wall thickening and rounding off at junctions of spore compartments
Rounding up of immature spores
Gray pigmentation, maturation and release of spores

eventually transformed into chains of pigmented spores (Hardisson and Manzanal, 1976; McVittie, 1974). This development of aerial hyphae occurs in a nonaqueous environment where required nutrients are only available where the base of the hyphae meet the substrate mycelium (Chater and Merrick, 1979).

I.2 Gene Products Which Play an Important Role in Morphological and Physiological Differentiation

The isolation and characterization of developmental mutants that are blocked at various stages of differentiation have been paramount in the elucidation of the regulatory signals involved in the developmental pathway of *Streptomyces*.

Investigations with two classes of developmental mutants have contributed much to our knowledge of the events involved in the differentiation process in *Streptomyces*, these are the *bld* and *whi* mutants.

I.2.1 The *whi* Mutants

The formation of the gray spore pigment of *S. coelicolor* depends on genes which act at early stages of sporulation (Hopwood *et al.*, 1970). Mutants that produce aerial hyphae but fail to develop the pigmentation associated with mature spores are known as the *whi* (for white) mutants (Chater, 1993). The *whi* mutations appear to only affect the differentiation of aerial hyphae into spores. Mutations in *whiA*, *-B*, *-G*, *-H*, or *-I* loci prevent the occurrence of regularly spaced sporulation septa (Chater, 1972; McVittie, 1974). The temporal order of function of the *whi* genes is: *whiG/J* < *whiA/B* < *whiH* < *whiI* < *whiD/E* (Chater, 1975; Chater *et al.*, 1989).

The product of *whiG* is a sigma (σ) factor which shares identity with σ^D of *Bacillus subtilis* (Chater, 1993; Chater *et al.*, 1989) and σ^F in *Salmonella typhimurium* (Chater, 1993; Hughes *et al.*, 1993). Both σ^D and σ^F are sigma factors that are subject to regulation by anti-sigma factors. It has been suggested that the morphologically coupled release of σ^{WhiG} from the anti-sigma factor may be important in the initiation of sporulation events (Kelemen *et al.*, 1996; Losick and Shapiro, 1993) and that the transition from straight to curled growth of a hypha occurs soon after σ^{WhiG} RNA polymerase has become active (Flardh *et al.*, 1999). Other σ^{WhiG} targets include the sporulation regulatory gene, *whiH* (Ryding *et al.*, 1998) and a gene which encodes a putative glycine-betaine-binding protein which may contribute to turgor required for the growth of aerial hyphae (Flardh *et al.*, 1999; Tan *et al.*, 1998). WhiH resembles a DNA-binding regulatory protein that is responsive to carboxylate-containing intermediates of carbon metabolism (Ryding *et al.*, 1998), suggesting that WhiH senses a metabolic intermediate whose concentration changes during growth of aerial hyphae (Flardh *et al.*, 1999). The *whiA* gene encodes a protein of unknown function (Flardh *et al.*, 1999). The *whiB* gene encodes a polypeptide that contains a high proportion of charged residues and is thought to operate as a transcription factor (Chater, 1993; Davis and Chater, 1992; Ryding *et al.*, 1998). The *whiE* locus determines the synthesis of spore pigment (Chater, 1993) and its transcription is regulated by two divergent promoters that depend on *whiA, -B, -G, -H, -I,* and *-J* (Kelemen *et al.*, 1998). It has been demonstrated that transcription of *whiB, -G,* and *-E* coincides with the development of aerial mycelium (Soliveri *et al.*, 1992).

The onset of sporulation requires a shift to a different mode of septation and chromosome partitioning from that observed in vegetative hyphae (Schwedock *et al.*, 1997). FtsZ, the key cell division protein, assembles into a large number of rings (Schwedock *et al.*, 1997) which are the precursors of sporulation septa. It has been demonstrated that *whiB*, *-G*, and *-H* mutants cannot assemble ladders of *FtsZ* rings in aerial hyphae (Schwedock *et al.*, 1997).

The *whiI* locus encodes an atypical member of the response regulator family of proteins. It does not posses two of the residues usually found to be conserved in the conventional phosphorylation pocket, and it is not located adjacent to a potential sensor kinase gene (Ainsa *et al.*, 1999). The *whiI* promoter has been found to be temporally controlled with a maximum level being coincident with spore formation (Ainsa *et al.*, 1999).

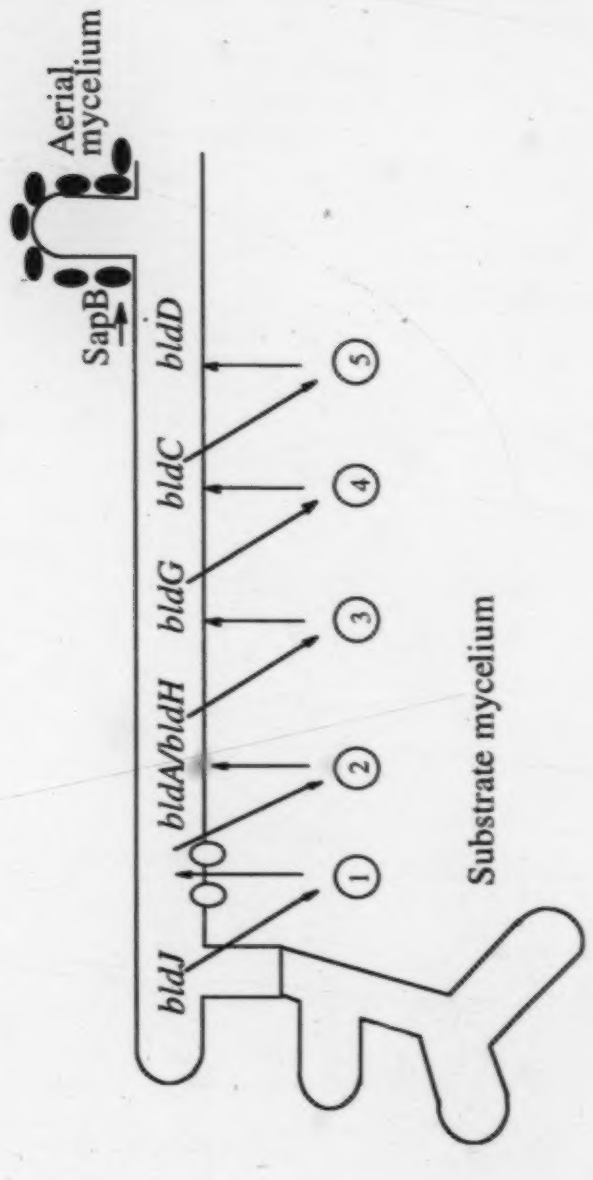
Five new sporulation loci have recently been determined in *S. coelicolor*: *whiK*, *whiL*, *whiM*, *whiN* and *whiO* (Ryding *et al.*, 1999). Characterization of these genes and their targets will cast new light on the regulatory cascade that controls sporulation in *S. coelicolor*.

I.2.2 The *bld* Mutants

The isolation of *bld* (for bald) mutants has played a pivotal role in the genetic characterization of aerial hyphae development. The *bld* mutants lack aerial mycelium and are shiny and 'bald' in appearance; a characteristic of vegetative growth (Merrick, 1976). Most of the *bld* mutants were selected for the bald phenotype on minimal medium containing glucose as carbon source or on a complex sporulation medium (Champness, 2000; Champness, 1988b). All of the well-characterized classes (except *bldB* and *bldI*) produce sporulating aerial mycelia when propagated on medium containing poorer carbon sources such as mannitol, galactose or maltose (Champness, 1988b; Merrick, 1976). Some of the *bld* mutants are also pleiotropically defective in antibiotic production (such as *bldA, bldD, bldG* and *bldH* (Champness, 1988b; Merrick, 1976) suggesting that the *bld* genes encode regulatory genes responsible for the onset of both morphological and chemical differentiation.

Extracellular complementation experiments in which pairs of *bld* mutants were grown on a rich medium in close proximity to one another indicated one mutant induces the other to synthesize SapB (see below), erect aerial hyphae and sporulate. This extracellular complementation is unidirectional with the following hierarchy: *bldJ* < *bldK* < *bldA/H* < *bldG* < *bldC* < *bldD* (Nodwell *et al.*, 1999; Willey *et al.*, 1993). Each mutant in the hierarchy can rescue the developmental defect in all the mutants to the left but not to the right. For example, the *bldD* mutant can complement all of the other mutants while *bldC*, which complements *bldJ, -K, -A, -H,* and *-G* cannot complement *bldD* (Figure I.2.3.1). This suggests that on a rich medium, aerial mycelium formation is initiated by a signalling cascade that involves at least five different extracellular signals culminating in SapB production (Nodwell *et al.*, 1996; Willey *et al.*, 1993). There are several lines of evidence which seem to indicate a more complex signalling cascade is involved than this model implies. For example, *bldB* and *bldI* do not fit into the above hierarchy (Willey *et al.*, 1993). In addition, the *bld* mutants are defective in the regulation of carbon utilization. As mentioned above, it has been demonstrated that *bldA, bldB, bldC, bldD,*

Figure I.2.2.1. A proposed regulatory signal cascade in *Streptomyces coelicolor*. Signals shown are produced by growth of substrate mycelium on a rich medium. The first extracellular factor (①) is produced by *bldJ* and is taken up by the BldK oligopeptide permease (⬯⬯) which triggers the synthesis and release of the second signal (②) and so on. The signal cascade eventually culminates in the *bldD*-dependent production of SapB (●) which coats the surface of the aerial mycelium. SapB facilitates the formation of aerial hyphae by reducing the surface tension at the interface between the air and the more aqueous environment of the substrate mycelium. Adapted from Kelemen and Buttner (1998).


Aerial mycelium
SapB
bldJ
bldA/bldH
bldG
bldC
bldD
①
②
③
④
⑤
Substrate mycelium

bldG and *bldH* are unable to either sense or signal starvation implying a deregulation of carbon utilization (Pope *et al.*, 1996). Furthermore, SapB is not detectable in cultures grown on minimal media containing either glucose or mannitol as carbon source (Willey *et al.*, 1991) suggesting an alternative pathway may be involved for growth on poorly utilized carbon sources (Champness and Chater, 1994).

It has been proposed, based on the extracellular complementation results, that the *bld* genes are involved, either directly or indirectly, in the production of the signalling molecules that are exchanged during morphogenesis. It has not been established if some of these signalling molecules are related to the γ-butyrolactones (see below). Since SapB restores only aerial hyphae formation, this suggests that it represents one of several developmental events blocked in *bld* mutants. To date, only *bldK and bldJ* appear to encode direct components of this signalling cascade (Nodwell and Losick, 1998; Nodwell *et al.*, 1996; Nodwell *et al.*, 1999).

The best characterized *bld* gene is *bldA* which encodes a leucyl tRNA that recognizes the UUA codon (Lawlor *et al.*, 1987), rare in *Streptomyces* mRNA because of the high G+C content of their DNA (70-74%) (Hopwood and Kieser, 1990). The TTA codon for leucine is confined to a very small number of *Streptomyces* genes primarily engaged in the regulation of morphogenesis and secondary metabolism (Distler *et al.*, 1992a; Fernández-Moreno *et al.*, 1991; Geistlich *et al.*, 1992; McCue *et al.*, 1992; Ueda *et al.*, 1993b) and appears to be absent from genes encoding essential proteins since it has been found to be dispensable for vegetative growth (Leskiw *et al.*, 1991a). The *bldA* gene product affects synthesis of many secondary metabolites (Fernández-Moreno *et al.*, 1991; Leskiw *et al.*, 1991a; White and Bibb, 1997). Although transcription of the *bldA* tRNA occurs from a vegetative promoter, the mature form of the tRNA does not accumulate until the switch to secondary metabolism, supporting a role for the tRNA in translational regulation of events late in growth (Leskiw *et al.*, 1991b; Leskiw *et al.*, 1993). Phenotypically similar *bldA* mutants in *S. lividans* and *S. griseus* suggest that this may be a general phenomenon in *Streptomyces* species (Champness and Chater, 1994).

Other *bld* genes include *bldB* (Elliott *et al.*, 1998) and *bldD* (Pope *et al.*, 1998) which are predicted to encode DNA-binding proteins which act as transcriptional regulators. The *bldG* gene encodes a protein product that has been shown to possess a high degree of similarity to *Bacillus subtilis* anti-anti-sigma factors (Bignell *et al.*, 2000). The function of the *bldI* gene has not been characterized but the level of *bldA* transcripts in *bldI* mutants were found to be significantly reduced indicating that *bldA* expression may be directly or indirectly regulated by *bldI* (Leskiw and Mah, 1995). The *bldK* locus encodes homologues of the polypeptide components of the ATP-binding cassette (ABC) membrane spanning transporters (Nodwell *et al.*, 1996) which likely function as an oligopeptide importer (Nodwell *et al.*, 1996) of *bldJ* (formerly *bld261*)-dependent, extracellular factor (Nodwell and Losick, 1998).

I.2.3 Other gene products are involved in sporulation

The process of sporulation involves the interplay of more than *bld* and *whi* gene products since not all of the developmental mutants correspond to these two categories. One such gene product is SapB (sporulation-associated protein), a small, morphogenetic protein which coats the surface of aerial hyphae (Willey *et al.*, 1991). This protein acts like a surfactant to facilitate the formation of aerial hyphae by reducing the surface tension at the interface between the air and the more aqueous environment of the substrate mycelium (Tillotson *et al.*, 1998). SapB production is temporally associated with production of aerial hyphae. It has been shown that aerial mycelium formation of *bldJ*,*-A*, *-B*, *-C*, *-D*, *-F*, *-G*, *-H*, *-I*, and *-K* can be restored by exogenous addition of SapB (Nodwell *et al.*, 1996; Willey *et al.*, 1991; Willey *et al.*, 1993) when these mutants are grown on a rich medium. Neither sporulation nor antibiotic production of *bld* mutants is restored (Tillotson *et al.*, 1998) by the addition of SapB. While production of SapB is impaired in *bld* mutants, production is normal in *whi* mutants (Willey *et al.*, 1991). Both aerial mycelium formation and sporulation can be restored to most *bld* mutants by growth on minimal medium containing mannitol as the carbon source (Champness, 1988). Under these conditions, antibiotic production is not restored and SapB is undetectable (Willey *et al.*, 1991).

The *ram* (rapid aerial mycelium) gene cluster consists of *ramA*, *ramB* and *ramR* (Ma and Kendall, 1994) and appears to regulate aerial mycelium formation independently of secondary metabolic functions (Ma and Kendall, 1994). The *ramA* and *ramB* genes encode proteins homologous to ATP-dependent membrane translocating proteins and it was found that disruption of the *ramB* gene resulted in defective aerial mycelium formation (Ma and Kendall, 1994). It has been proposed that RamA and RamB export a product from the cell with a candidate being SapB (Ma and Kendall, 1994) although there is currently no direct evidence for this. The *ramR* gene encodes a protein with significant homology to the UhpA subset of bacterial two-component response regulator proteins (Ma and Kendall, 1994) which are known to play a role in sugar-phosphate transport in *E. coli* (Stock *et al.*, 1989). Unlike most two-component response regulators, a sensor kinase is not present in the same operon as *ramR* and may indicate that it represents a final step in a larger network of regulators and sensors that controls differentiation (Ma and Kendall, 1994). Since none of the *ram* genes contain a TTA codon, it appears that the inability of *bldA* mutants to synthesize aerial mycelia is not due to an inability to translate the *ram* genes (Ma and Kendall, 1994).

Mutations in the adenylate cyclase gene, *cya*, also exhibit a bald phenotype. These mutants were found to irreversibly acidify the growth medium suggesting that the onset of differentiation involves a shift in metabolism whereby the excreted organic acids are taken up and utilized (Süsstrunk *et al.*, 1998) (see I.4.3).

I.3 Antibiotic Production in *S. coelicolor*

S. coelicolor produces at least four antibiotics. Undecylprodigiosin (Red), a red-pigmented antibiotic (or yellow, depending on pH), is a cell-associated, nonpolar tripyrrole and is the major component of a mixture of three prodigionines (undecylprodigiosin, butylcycloheptylprodiginine, and dipyrrolyldipyrromethane) (Tsao *et al.*, 1985). It is chemically similar to the antibiotic prodigiosin produced by *Serratia marcescens* (Tsao *et al.*, 1985). The production of undecylprodigiosin occurs in the transition and stationary phases of liquid-grown cultures (Takano *et al.*, 1992) and coincides with the onset of morphological development in surface-grown culture (Feitelson and Hopwood, 1983; Rudd and Hopwood, 1980). On most laboratory media, *bldA* mutants do not produce undecylprodigiosin (Guthrie and Chater, 1990). The two pathway-specific regulators (ie: regulators linked to antibiotic biosynthetic gene clusters to control their transcription), RedD (Guthrie and Chater, 1990) and RedZ (White and Bibb, 1997), regulate the ~36 kb biosynthetic cluster for undecylprodigiosin (Malpartida *et al.*, 1990). *redZ* encodes a homolog of the response regulator family of proteins but it lacks several of the conserved amino acid residues that form the phosphorylation pocket, including the aspartate residue that is phosphorylated by the sensor kinase (Guthrie *et al.*, 1998). The transcription of *redZ* is not *bldA*-dependent, however, since it contains a UUA codon, translation of *redZ* mRNA is (White and Bibb, 1997).

Actinorhodin, a blue-pigmented antibiotic (blue in alkali and red in acid) which diffuses into the media surrounding the colonies (Wright and Hopwood, 1976b), belongs to the anthracycline class of antibiotics which includes the anti-cancer therapeutic agents daunorubicin and doxorubicin (Arcamone *et al.*, 1969; Di Marco *et al.*, 1964). Actinorhodin, a polyketide antibiotic, has sparked much interest since many commercially important antibiotics are polyketides (Hopwood and Sherman, 1990). It is synthesized through a series of condensations of carbon units in a manner similar to that of long chain fatty acid biosynthesis (Hopwood and Sherman, 1990). The *act*II region encodes the pathway specific activator, *actII*-ORF4, as well as the export genes for the 25 kb actinorhodin biosynthetic cluster (Fernández-Moreno *et al.*, 1991). As is the case for undecylprodigiosin (Red), the loss of production of actinorhodin (Act) in *bldA* mutants can be accounted for by the presence of a TTA codon in the pathway-specific activator for Act (*act*II-ORF4) (Fernández-Moreno *et al.*, 1991).

The biosynthetic gene cluster for the third antibiotic, methylenomycin, a cyclopentanone, is located on the linear SCP1 plasmid (Kinashi and Shimaji-Murayama, 1991; Kinashi *et al.*, 1992; Wright and Hopwood, 1976a) and represents the only example of an entire antibiotic cluster mapping to an extrachromosomal element in *Streptomyces* (Champness, 2000; Hopwood and Wright, 1983).

The fourth antibiotic produced by *S. coelicolor* is the calcium-dependent antibiotic (CDA) and has been the least studied. CDA is a peptide antibiotic which is synthesized non-ribosomally by a putative multi-functional peptide synthetase. The 35 kb cluster has been sequenced (Chong *et al.*, 1998). A pathway specific regulator, CdaR, is similar to ActII-Orf4, RedD, and AfsR (http://www.sanger.ac.uk).

Mutants of the Abs$^-$ phenotypic class are incapable of producing the four antibiotics just described yet are still able to sporulate (Adamidis and Champness, 1992; Adamidis *et al.*, 1990). These mutants can be divided into two classes consisting of *absA* and *absB*. The *absA* locus encodes a sensor histidine kinase (AbsA1) and response regulator (AbsA2) (Brian *et al.*, 1996). Disruption of this locus resulted in precocious overproduction of antibiotics indicating it negatively regulates antibiotic production (Brian *et al.*, 1996). It is likely that the *absA* mutants are unable to respond to environmental signals and relieve repression of antibiotic production (Brian *et al.*, 1996). The *absB* locus encodes a homologue of RNaseIII (Aceti and Champness, 1998; Brian *et al.*, 1996) but its role in antibiotic production is still unclear. Surprisingly, AbsA1/AbsA2, which appear to be global regulators of antibiotic production, are located in the *cda* cluster and are divergently transcribed from *cdaR*, the pathway-specific regulator for CDA. It has been proposed that the AbsA1/AbsA2 pair may be directly involved in the regulation of *cdaR* with AbsA2~P acting as a repressor of *cdaR*, *redZ*, and *actII*-orf4 (W. Champness, personal communication).

I.3.1 Diffusible Signalling Molecules

Signalling molecules known as γ-butyrolactones are hormone-like autoregulators structurally similar to homoserine lactones involved in quorum sensing in Gram negative bacteria (Fuqua *et al.*, 1994). For example, they act as inducers for bioluminescence in *Vibrio harveyi* (Cao and Meighen, 1989) and *Vibrio fischeri* (Eberhard *et al.*, 1981), the transfer of the T_i plasmid in *Agrobacterium tumefaciens* (Fuqua *et al.*, 1994; Zhang *et al.*, 1993) and they act as inducers for the production of exoenzyme and antibiotics in *Erwinia carotovora* (Bainton *et al.*, 1992). The γ-butyrolactone autoregulators act by binding to specific receptor proteins to modulate transcription. The microbial hormone A-factor (2-isocapryloyl-3R-hydroxymethyl-γ-butyrolactone) has been found to be essential for streptomycin (Sm) biosynthesis and aerial mycelium formation in *S. griseus* (Horinouchi and Beppu, 1994; Miyake *et al.*, 1990). An *S. griseus* mutant, deficient in A-factor, does not produce aerial mycelium and displays a bald phenotype. Restoration of aerial mycelium formation, sporulation and streptomycin production is possible with exogenous supplementation of A-factor at a concentration of 1 nM (Ueda *et al.*, 1993b; Ueda *et al.*, 1996). A-factor is able to cross the cell membrane by diffusion to complex with a cytoplasmic repressor-type receptor protein ArpA (A-factor receptor protein) thereby dissociating it from the promoter region of *adpA*. The resultant AdpA (A-factor-dependent protein), an A-factor responsive transcriptional activator, binds the promoter of *strR*, a pathway-specific regulatory gene which activates transcription of other streptomycin biosynthesis genes (Ohnishi *et al.*, 1999). ***Streptomyces coelicolor*** is capable of cross-feeding *S. griseus* mutants that are deficient in A-factor synthesis (Hara *et al.*, 1983) and produces at least four small, diffusible signalling molecules that can cause precocious antibiotic production. So far only one of these signals, ScbI, is known to be a γ-butyrolactone (Champness, 2000; Kawabuchi *et al.*, 1997). Since *S. coelicolor* does not possess an A-factor receptor protein, A-factor does not appear to behave as an autoregulator in this organism (Miyake *et al.*, 1989). Interestingly, a TTA-containing *adpA* homologue has recently been identified on cosmid StC105 in *S. coelicolor* (http://www.sanger.ac.uk). This suggests that *S. coelicolor* and *S. griseus* share common regulatory elements for antibiotic production and morphological differentiation. Two genes, *cprA* and *cprB* (*coelicolor* pigment regulator), each encoding an ArpA-like protein have been identified in *S. coelicolor* (Onaka *et al.*, 1998). Disruption of *cprA* resulted in reduction of actinorhodin and undecylprodigiosin production and delayed sporulation, indicating that this gene acts as a positive regulator for secondary metabolite formation and aerial mycelium formation (Onaka *et al.*, 1998). While disruption of the *crpB* gene resulted in earlier sporulation and precocious overproduction of actinorhodin, it appeared to have no effect on undecylprodigiosin indicating it was likely a negative regulator of actinorhodin production and aerial mycelium formation (Onaka *et al.*, 1998). The discovery of CprA and CprB adds another layer of complexity to what already appears to be a complicated regulatory system governing secondary metabolism and morphogenesis in *Streptomyces*.

Virginiae butanolides were isolated as autoregulators controlling virginiamycin production in *S. virginiae* (Yamada *et al.*, 1987), and screens for A-factor-like autoregulators led to the isolation of Factor I from *S. viridochromogenes* (Grafe *et al.*, 1982) and A-factor-like homologues from *S. bikiniensis* and *S. cyaneofuscatus* (Grafe *et al.*, 1983; Horinouchi and Beppu, 1994). These findings led to the assumption that A-factor homologues in streptomycetes play a general role in the regulation of secondary metabolism and morphological differentiation (Horinouchi and Beppu, 1994).

Other examples of diffusible signalling molecules have been identified but are less characterized. Factor C, a 34 kDa protein capable of restoration of sporulation to a nonsporulating *S. griseus* mutant (Birko *et al.*, 1999; Biro *et al.*, 1980) appears to function as a microbial hormone. Pamamycin, produced by *S. ambofaciens* (McCann and Pogell, 1979) and *S. alboniger* (Kondo *et al.*, 1988) provides the only confirmed example of a compound that plays a developmental role in the producing organism and also has antibiotic activity. A *bldJ*-dependent extracellular factor produced by *S. coelicolor* has been isolated but not characterized (Nodwell and Losick, 1998).

The mechanisms governing the coordinate regulation of morphogenesis and antibiotic production have not yet been elucidated. Investigations into how these diffusible signalling molecules are able to exert an intracellular as well as intercellular effect will aid in the understanding of the complex regulatory network that controls the developmental pathway in *Streptomyces*.

I.4 Nutritional and Physiological Factors That Affect Development

I.4.1 Metals, Cofactors and Metabolism

Microbial growth requires many heavy metals in low concentrations (Doelman, 1986) while at high concentration, some of these metals may be toxic (Gadd and Griffiths, 1978). Metals such as cadmium and mercury which do not have a function in cellular metabolism are extremely toxic even at low concentration (Vallee and Ulmer, 1972). Heavy metals can facilitate the inhibition of enzymatic activities and the precipitation of nutrients such as phosphate which in turn influences their accessibility to the microbe (Doelman, 1986). Some metals are known to be important for secondary metabolite formation and cell differentiation since they are involved in the activation of some biosynthetic pathways (Ueda *et al.*, 1993a; Weinberg, 1970; Weinberg, 1977a; Weinberg, 1977b).

Iron is a vital but potentially toxic metal and its metabolism is tightly regulated (Gruer and Guest, 1994; Leoni *et al.*, 1996). Iron-containing enzymes are known to participate in the sporulation of *B. subtilis* (Alen and Sonenshein, 1999) and *Streptomyces* (Schwartz *et al.*, 1999).

The ions of some metals such as iron, magnesium, copper, manganese, cobalt, zinc and calcium behave as cofactors. The WhiB-WhiD family of proteins in *S. coelicolor*, share conservation of four cysteines which have been proposed to act as ligands for a metal cofactor (Molle *et al.*, 2000). The aconitase proteins of *S. viridochromogenes* and *B. subtilis* have a 4Fe-4S cluster at the catalytic site (Alen and Sonenshein, 1999; Schwartz *et al.*, 1999).

Calcium is important in both morphological and biochemical differentiation in *Streptomyces*. It is essential in triggering spore germination and a high calcium content is observed in *Streptomyces* spores (Eaton and Ensign, 1980; Salas *et al.*, 1983). Calcium ions have been shown to induce aerial mycelium formation in *S. ambofaciens* (Fernández-Moreno *et al.*, 1991). The secretion of extracellular enzymes was found to increase when high concentrations of calcium were supplied in the growth medium (Kamel *et al.*, 1989). The production of actinorhodin was also found to be affected by calcium concentration (Abbas and Edwards, 1990). Manganese and calcium are also necessary in sporulation and secondary metabolite formation in *Bacillus subtilis* (Ueda *et al.*, 1993a). It has been observed that some tyrosine kinases require a manganese ion for the phosphorylation reaction (Distler *et al.*, 1992b). Investigations into the effect of metals on growth and actinorhodin production (Abbas and Edwards, 1990) found that manganese and calcium, when added separately, enhanced growth but caused reduced antibiotic titers.

I.4.2 Storage Compounds as Energy Reserves

Intracellular storage compounds such as glycogen and poly-β-hydroxybutyrate have been found to accumulate in most bacteria subjected to nutrient conditions that limit growth (Dawes, 1992) and *Streptomyces* is not an exception. Polyhydroxybutyrate accumulates during exponential growth of *S. venezualae* but disappears from mycelium in stationary phase (Ranade and Vining, 1993). The depletion of polyhydroxybutyrate is coincident with chloramphenicol production (Ranade and Vining, 1993) in this organism but was not used as a precursor for antibiotic production suggesting it may have a role as an energy reserve.

Glycogen, a large polymer of α-1,4-linked glucose residues with α-1,6-linked branches, is temporally associated with differentiation and is detected in sporulating aerial hyphae but absent in mature spores (Brana *et al.*, 1986; Karandikar *et al.*, 1997). Glycogen gradually disappears during maturation suggesting it is involved in synthesis of some spore components or is a source of energy to complete the sporulation process (Brana *et al.*, 1986; Plaskitt and Chater, 1995; Ranade and Vining, 1993). Karandikar *et al.* (1997) observed that intracellular glycogen was degraded while glucose was still present in the medium suggesting that either enzymes involved in glycogen degradation are insensitive to catabolite repression or that glycogen utilization in some parts of the mycelium occurs spatially separate from glucose utilization in other parts of the mycelium.

Trehalose accumulation has also been observed in many *Streptomyces* species (Ensign, 1982; Nimi *et al.*, 1984). Trehalose was found to accumulate in regions of the colony farthest from the hydrated medium leading Brana *et al.* (1986) to suggest it may play a role in protecting the aerial mycelium and perhaps even the spores from dessication. This disaccharide has been shown to act as a carbon storage compound in *S. griseus* spores (McBride and Ensign, 1987a; McBride and Ensign, 1987b).

Streptomyces have been shown to use triacylglycerols as storage compounds and it has been speculated that these serve as possible carbon sources for antibiotic biosynthesis (Olukoshi and Packter, 1994). These triacylglycerols are accumulated intracellularly in membrane-bound structures during the exponential growth phase (Packter and Olukoshi, 1995). The utilization of these compounds would require lipolytic enzymes. It has been demonstrated that lipase synthesis in *S. lividans* 66 carrying the *S. exfoliatus lipA* gene is growth-phase dependent and that transcription from the *lipA* promoter was dependent on the presence of the contiguous downstream gene, *lipR*. The *lipR* gene possesses a TTA codon which causes *bldA*-dependence of *lipA* transcription (Servin-Gonzalez *et al.*, 1997). While *bldA* involvement in triacylglycerol utilization seems clear, the signals that regulate the formation and degradation of these storage compounds and how they are linked to biochemical and morphological differentiation are at present unknown.

I.4.3 Carbon Utilization, Catabolite Control and the Role of cAMP

In nature, the primary sources of carbon for the soil dwelling *Streptomyces* are complex carbohydrates such as cellulose, chitin and xylan which comprise the major constituents of soil (Delic *et al.*, 1992). Currently, knowledge about how *S. coelicolor* senses nutrient levels, transports carbohydrates into the cell and how it regulates the utilization of carbohydrate substrates is limited. How these pathways influence secondary metabolite production and differentiation also remains to be elucidated.

The phosphotransferase system (PTS) catalyzes the accumulation of carbohydrates as phosphorylated derivatives (Medow *et al.*, 1990; Postma *et al.*, 1993). The phosphoryl donor is phosphoenolpyruvate (PEP), a glycolytic intermediate (Postma *et al.*, 1993). In *B. subtilis*, HPr and HPr kinase/phosphatase play pivotal roles in carbon catabolite repression (Hueck and Hillen, 1995; Parche *et al.*, 1999; Reizer *et al.*, 1993). HPr is phosphorylated on a serine residue by HPr kinase/phosphatase and acts as a corepressor of catabolite-controlled genes (Deutscher *et al.*, 1995; Parche *et al.*, 1999; Reizer *et al.*, 1993). HPr carbon catabolite repression can be exerted by various carbohydrates such as fructose, glucose, and mannitol (Kwakman and Postma, 1994). The signal transduction pathway leading to carbon catabolite repression in *S. coelicolor* is still not understood in detail. A protein homologue of the *Bacillus* phosphocarrier protein, HPr, has recently been identified in *S. coelicolor* (Parche *et al.*, 1999). The adjacent genes, which in other bacteria encode additional proteins of the PTS, are not present in *S. coelicolor* (Parche *et al.*, 1999). Whether HPr in *S. coelicolor* plays a regulatory role is at present unclear since an HPr kinase/phosphatase has not been detected in this organism. Since HPr is an important component of the regulation of carbon utilization in other bacteria, investigations into the regulatory role it may play in *S. coelicolor* will aid in the understanding of how carbon utilization influences secondary metabolite production and morphological differentiation.

The best studied system for catabolite repression is the cyclic adenosine 3',5'-monophosphate (cAMP)-dependent system of *E. coli*. In this Gram negative organism, cAMP levels are high when catabolite levels are low, as when the organism is grown on a poor carbon source (Ishizuka *et al.*, 1993). The synthesis of cAMP is controlled through the regulation of adenylate cyclase activity. Adenylate cyclase (*cya*) makes cAMP from ATP and is more active when cellular concentrations of catabolites are low and less active when catabolite concentrations are high (Botsford and Harman, 1992). Studies investigating catabolite control in *S. coelicolor* suggest that glucose kinase and not cAMP is required for catabolite control (Chatterjee and Vining, 1982a; Chatterjee and Vining, 1982b; Hodgson, 1982) yet not all genes that are subject to carbon catabolite control depend on glucose kinase (Ingram and Westpheling, 1995; Walter and Schrempf, 1996). It is interesting that cAMP does not seem to be the effector molecule in carbon catabolite repression in *B. subtilis* either (Fisher and Sonenshein, 1991).

The *S. coelicolor bld* mutants have been shown to be defective in the regulation of carbon utilization. There has been suggestion that cAMP exerts general pleiotropic effects on secondary metabolism and morphological development in *Streptomyces* spp. Ragan and Vining (1978) observed that there was an indirect relationship between cAMP and streptomycin biosynthesis. More recently, it has been determined that distinct phenotypic changes are due to the effect exerted by cAMP (Kang *et al.*, 1999; Süsstrunk *et al.*, 1998). Süsstrunk *et al.* (1998) demonstrated pleiotropic effects of cAMP on secondary metabolism and cell differentiation in *S. coelicolor* A3(2). In *Streptomyces* species, cAMP behaves as a second messenger (Süsstrunk *et al.*, 1998). Adenylate cyclase mutants are unable to synthesize cAMP (*cya* mutants), cannot neutralize the medium which becomes acidified during mycelium formation, and exhibit a bald phenotype on unbuffered medium. This phenotype can be suppressed by the addition of exogenous cAMP. It was also demonstrated that wild type *S. coelicolor* accumulated cAMP prior to the onset of aerial mycelium formation and secondary metabolism suggesting that cAMP is required for these processes. In *S. coelicolor*, a decrease in pH due to acid accumulation in a *cyaA*-disrupted strain was found to influence antibiotic biosynthesis and morphological development (Süsstrunk *et al.*, 1998). Although an accumulation of organic acids in the medium results in a bald phenotype it is unclear whether the defect in carbon utilization is responsible. It has been hypothesized that cAMP mediates a switch in metabolism from an acid-producing phase to an acid utilizing phase which is associated with differentiation (Süsstrunk *et al.*, 1998).

When wild type *S. coelicolor* is grown on solid glucose minimal medium, organic acids are excreted during the phase of growth that generates substrate mycelium. When aerial mycelium is produced, a shift in metabolism occurs so that the medium becomes neutralized; probably due to the uptake and reutilization of extracellular acids (Liu *et al.*, 1985; Süsstrunk *et al.*, 1998). The *bldA, bldB, bldC, bldD, bldG* and *bldH* mutants are not only unable to signal starvation (Pope *et al.*, 1996) but they also irreversibly acidify their growth medium. Unlike the *cya* mutant, the *bld* mutants are not rescued by growth on medium containing neutralizing buffer nor by exogenous addition of cAMP (Süsstrunk *et al.*, 1998). This suggests that although a metabolic imbalance results as a consequence of many *bld* mutations, current knowledge of *Streptomyces* primary metabolism is limited and therefore it is difficult to determine whether or not the metabolic shift serves as a general developmental trigger (Karandikar *et al.*, 1997; Süsstrunk *et al.*, 1998).

I.4.4 GTP Levels

A decrease in intracellular GTP concentration can occur in response to nutrient limitation and may serve as a signal to trigger differentiation. In eukaryotes, GTP-binding proteins have been found to participate in signal transduction processes such as the regulation of hormones and the regulation of adenylate cyclase (Gilman, 1987; Kaziro *et al.*, 1991). In *B. subtilis*, a GTP-binding protein (Obg), has been found to be involved in the initiation of sporulation. A homologous protein in *S. coelicolor* (also designated Obg) has been demonstrated to have an essential function in vegetative growth and when present in high copy was found to enhance aerial mycelium formation and antibiotic production (Obaya and Guijarro, 1993; Okamoto and Ochi, 1998). It has been demonstrated (Lopez *et al.*, 1981; Ochi, 1987a; Ochi, 1987b; Ochi *et al.*, 1981) that a decrease in GTP pools correlates with the initiation of morphological differentiation in *B. subtilis* and *Streptomyces* species. A role for GTP-binding proteins in sensing decreased GTP levels as a signal for differentiation has been proposed (Itoh *et al.*, 1996), however no clear evidence for this has yet been uncovered.

I.4.5 Stringent Response

It has been observed that a slow growth rate or the cessation of growth acts as a signal for antibiotic production (Chater and Bibb, 1997). Morpholgical and physiological differentiation is believed to be a response, at least in part, to nutrient limitation. The stringent response is a regulatory system coupled to nutrient limitation which causes cessation of RNA synthesis (Cashel, 1975; Gallant, 1979). The stringent response factor, ppGpp (guanosine 5'-diphosphate 3'-diphosphate), is synthesized in response to nutrient conditions such as nitrogen limitation and amino acid starvation (Chakraburtty and Bibb, 1997). Developmental gene expression in *Myxococcus xanthus* leading to multicellular fruiting body formation is induced by ppGpp synthesized in response to amino acid limitation (Harris *et al.*, 1998). In *Escherichia coli*, ppGpp has been shown to play a role in the control of gene expression and growth rate (Glass *et al.*, 1986; Hernandez and Bremer, 1990). In the case of amino acid starvation, the *relA* gene product, a (p)ppGpp synthetase (Pedersen and Kjeldgaard, 1977) synthesizes ppGpp. ppGpp is synthesized from ATP and GTP by the ribosome-bound RelA that is activated by uncharged tRNAs binding to the activator site of translating ribosomes (Cashel *et al.*, 1996).

The *relA* gene has been cloned from *S. coelicolor* (Chakraburtty *et al.*, 1996). In streptomycetes, it has been proposed that accumulation of ppGpp and pppGpp (guanosine 5'-triphosphate 3'-diphosphate) results in antibiotic production (Ochi, 1986; Ochi, 1987b; Ochi, 1990a; Ochi, 1990b), while morphological differentiation results from a decrease in the GTP pool (Ochi, 1990a). Under nitrogen limitation, *S. coelicolor relA* mutants are unable to produce actinorhodin or undecylprodigiosin and exhibit a delay in the onset of morphological differentiation (Chakraburtty and Bibb, 1997). One of the effects of stringent response is inhibition of transcription from stringently controlled promoters such as those responsible for the transcription of rRNA and tRNA genes (Staunch *et al.*, 1991). A positive correlation has been found between ppGpp synthesis and *redD* transcription (Bibb, 1996a; Takano *et al.*, 1992) as well as *act*II-ORF4 transcription (Staunch *et al.*, 1991) during the transition and stationary phases of *S. coelicolor* grown in liquid cultures. This could be an indirect effect of growth cessation rather than a direct result of ppGpp level. It has been proposed (Ochi, 1987b) that the intracellular accumulation of ppGpp triggers the biosynthesis of streptomycin in *S. griseus* and that a decrease in the GTP pool leads to the formation of aerial mycelium. However, Bascaran *et al.* (1991) did not find a relationship between ppGpp and antibiotic production in *S. clavuligerus*. In their experiments, cephalosporin production was initiated at the end of exponential growth and increased during stationary phase while levels of ppGpp remained constant throughout growth.

S. coelicolor relC mutants, devoid of the ribosomal protein ST-L11 which interacts with (p)ppGpp synthetase, exhibited delayed sporulation and actinorhodin production and were unable to produce undecylprodigiosin (Ochi, 1990a). The *relA* and *relC* mutations both exert their effect at the ribosomal level leading to an inability of cells to accumulate ppGpp. Investigations by Shima *et al.* (1996) demonstrated that altered ribosomal protein S12 resulting from mutations in the *rspL* gene, which conferred resistance to streptomycin, was able to activate actinorhodin production in *S. lividans* as well as bypass the effects of the *relA* mutation in *S. coelicolor*. This led to the suggestion that the role of ppGpp in the initiation of secondary metabolism may be to modify a ribosomal component upon binding which results in changes in the translational function of the ribosome (Shima *et al.*, 1996).

Although correlations between ppGpp synthesis and the initiation of antibiotic production have been observed it does not appear to have a general role in the initiation of antibiotic production between different streptomycetes. It is likely that ppGpp plays a role in triggering the onset of antibiotic production but it is not always sufficient in that some biosynthetic pathways must depend on additional factors that remain to be identified.

I.4.6 Nitrogen Metabolism

Nitrogen, a component of many biological molecules including amino acids, vitamins and nucleotides, is required for bacterial growth and can be supplied by amino acids, ammonia and nitrate. Nitrogen sources which support low growth rates have been found to be favorable for antibiotic synthesis (Omura *et al.*, 1983). The available nitrogen source can affect enzymes required for the synthesis of precursors for antibiotic biosynthesis (Omura *et al.*, 1983). Investigations into the mechanism mediating preferences in nitrogen source utilization have resulted in different models of nitrogen control with the enzyme responsible for ammonium assimilation, glutamine synthetase, and the product of ammonium assimilation, glutamine, thought to play key roles (Fisher, 1992). Ammonium has been found to repress glutamine synthetase and antibiotic production. This repression is reversed when ammonium is exhausted from the growth medium suggesting a role for glutamine synthetase in nitrogen control of antibiotic production (Aharonowitz and Demain, 1979).

In nitrate-limited conditions, *Streptomyces coelicolor* exhibits a biphasic growth pattern (Karandikar *et al.*, 1997). The pH of the medium changes throughout the different growth phases with an initial increase in pH during rapid growth followed by a decrease in pH suggesting the presence of organic acids in the medium (as observed by Süsstrunk *et al.*, 1998; Liu *et al.*, 1985 and Redshaw *et al.*, 1976). Under nitrogen exhaustion, glycogen accumulates (Karandikar *et al.*, 1997; Ranade and Vining, 1993) and hydrophobicity of the organism increases (Karandikar *et al.*, 1997) which is not surprising given the hydrophobic nature of spores. Following nitrate depletion, triacylglycerols accumulate (Karandikar *et al.*, 1997) and probably serve as an energy reserve. Nitrate exhaustion is linked to aerial hyphae formation (Karandikar *et al.*, 1997). A stringent response may be elicited from exhaustion of nitrogen having an effect on RNA and protein synthesis.

The complex interplay of signals generated by carbon utilization, pH, ppGpp pools, cAMP and nitrogen availability all influence gene expression and translation. How these diverse signals are coordinated remains to be determined.

I.5 Role of Phosphorylation in the Regulation of Physiological Functions

Phosphorylation adds yet another layer of complexity to the elaborate network of regulatory signals involved in differentiation in *Streptomyces*. Reversible phosphorylation of proteins is now recognized to be a major mechanism for the control of intracellular events of cells (Bourret *et al.*, 1991; Stock *et al.*, 1989). Processes as diverse as metabolism, membrane transport, secretion, cell division, transcription and translation of genes are all regulated by this post-translational modification (Stock *et al.*, 1990).

Based on sequence similarity and enzymatic specificity, there are two superfamilies of protein kinases (Zhang, 1996): the histidine kinase superfamily (Stock *et al.*, 1990) and the superfamily of serine, threonine, and tyrosine kinases (Hanks *et al.*, 1988; Hunter, 1995).

I.5.1 Two-Component Signal Transduction

The expression of a variety of bacterial genes is controlled by two-component regulatory systems (Bourret *et al.*, 1991; Parkinson, 1993; Stock *et al.*, 1989). It is generally believed that two-component systems are used to sense environmental levels of essential substances for growth such as nitrogen, phosphate, and carbon source availability as well as molecules that signal changes in environmental conditions that require an adaptive response (Bourret *et al.*, 1991; Stock *et al.*, 1994; Stock *et al.*, 1990). In general, these systems contain a pair of proteins referred to as sensors and response regulators. The genes for these components are usually found adjacent to each other. The sensor proteins are histidine protein kinases that phosphorylate or dephosphorylate their cognate response regulators in response to environmental stimuli (Parkinson, 1993; Parkinson and Kofoid, 1992; Perego and Hoch, 1996). These sensors are generally integral membrane proteins (Bourret *et al.*, 1991; Parkinson and Kofoid, 1992; Stock *et al.*, 1989). The histidine kinase receives a signal at its amino-terminal domain and is autophosphorylated at the conserved histidine residue in an ATP-dependent reaction (Bourret *et al.*, 1991; Parkinson and Kofoid, 1992). The carboxy-terminal domain of the kinase then transduces the signal by transfer of the phosphoryl group to an aspartate residue in the amino-terminal domain of the second component, the response regulator

(Parkinson, 1993; Parkinson and Kofoid, 1992). The response regulator often functions as a trancriptional activator. Response regulators have at least three conserved residues: two aspartates and one lysine (Parkinson and Kofoid, 1992; Stock *et al.*, 1989; Stock *et al.*, 1990). Phospho-histidine is a high-energy molecule that can be quickly dephosphorylated by hydrolysis (Stock *et al.*, 1990). Protein histidine kinases were once believed to exist only in prokaryotes, however, proteins with homology to the bacterial histidine kinases have been identified in eukaryotes including animals, fungi and plants (Alex and Simon, 1994; Brown *et al.*, 1994; Chang *et al.*, 1993; Hughes, 1994; Ota and A., 1993; Popov *et al.*, 1992; Swanson *et al.*, 1994).

A few natural inhibitors of two-component regulatory systems have been reported. For example, unstaturated fatty acids are non-competitive inhibitors of ATP-dependent autophosphorylation of the histidine protein kinase, KinA, involved in the regulation of sporulation in *B. subtilis* (Strauch *et al.*, 1992). Oleic acid inhibits the formation of KinA-phosphate in a concentration-dependent manner in the presence or absence of the cognate response regulator but does not inhibit bacterial growth (Strauch *et al.*, 1992). No such inhibitors have been reported for *Streptomyces.*

The role of phosphorylation in the regulation of secondary metabolism and cell differentiation in *Streptomyces* is not very well understood, but is implicated in activating antibiotic synthesis involving the two component histidine kinase-response regulator family (Table I.5). To date, there are three examples of two-component- type signal transduction systems in *S. coelicolor*. Both the AbsA1/AbsA2 (Brian *et al.*, 1996) and CutR/CutS (Chang *et al.*, 1996) pairs act to negatively regulate antibiotic production while AfsQ1/AfsQ2 (Ishizuka *et al.*, 1992) exert a positive effect on antibiotic

Table I.5. Phosphorylated Signalling Proteins in *Streptomyces* spp.

Gene Product	Comment	Reference
AbsA1	Two-component sensor kinase	(Brian *et al.*, 1996)
AbsA2	Two-component response regulator	(Brian *et al.*, 1996)
AfsK	Membrane-bound, phosphorylates AfsR; autophosphorylates on Ser and Tyr residues; Ser-Thr protein kinase	(Matsumoto *et al.*, 1994)
AfsQ1	Two-component response regulator; cloned from *S. coelicolor*	(Ishizuka *et al.*, 1992)
AfsQ2	Two-component sensor kinase; cloned from *S. coelicolor*	(Ishizuka *et al.*, 1992)
AfsR	Functions as transcription regulator; phosphorylated by membrane bound kinase AfsK; phosphoryated on Ser and Thr residues; related to Ser-Thr phosphoproteins common to eukaryotic signal transduction pathways	(Hong *et al.*, 1991); (Horinouchi and Beppu, 1992); (Matsumoto *et al.*, 1994)
AmfR	Two-component response regulator; contains a TTA codon; putative *bldA* target; isolated from *S. griseus*	(Ueda *et al.*, 1993)
BrpA	Pathway specific regulator of biolaphos; weakly resembles response regulators but lacks the conserved phosphorylation motif; not associated with a cognate kinase	(Raibaud *et al.*, 1991)
CseB	Two-component response regulator; control of *sigE*; isolated from *S. coelicolor*	(Paget *et al.*, 1999)
CseC	Two-component sensor kinase; control of *sigE*; isolated from *S. coelicolor*	(Paget *et al.*, 1999)
CutR	Two-component response regulator; involved in copper metabolism	(Chang *et al.*, 1996)
CutS	Two-component sensor kinase; involved in copper metabolism	(Chang *et al.*, 1996)
DnrN	Pseudo response regulator; not associated with a sensor kinase; *dnrN* essential for transcription of *dnrI* regulatory gene (DnrI is similar to AfsR, RedD, and *act*II-ORF4)	(Otten *et al.*, 1995); (Madduri and Hutchinson, 1995)
GlnR	Response regulator; differs from other regulators at certain highly conserved residues; is not adjacent to a sensor kinase; isolated from *S. coelicolor*; positively regulates the transcription of *glnA* (glutamine synthetase)	(Wray and Fisher, 1993)
PkaA	Kinase; phosphorylated on Thr and Ser	(Urabe and Ogawara, 1995)
PkaB	Kinase; phosphorylated on Thr	(Urabe and Ogawara, 1995)
Pkg2	Transmembrane protein Ser/Thr kinase; isolated from *S. granaticolor*	(Nadvornik *et al.*, 1999)
Pkg3	Protein Ser/Thr kinase; does not undergo autophosphorylation; contains a TTA codon; putative *bldA* target	(Vomastek *et al.*, 1998)
Pkg4	Protein Ser/Thr kinase; does not undergo autophosphorylation; contains a TTA codon; putative *bldA* target	(Vomastek *et al.*, 1998)
RamR	Homologous to UhpA two-component response regulators; not associated with a sensor kinase	(Ma and Kendall, 1994)
WhiI	Atypical response regulator; does not possess conserved amino acids in phosphorylation pocket; not associated with a sensor kinase	(Ainsa *et al.*, 1999)

production. The signals that are sensed by the sensor kinases in each of these pairs have yet to be identified.

I.5.2 Phosphorylation on Serine, Threonine and Tyrosine

For a long time it was believed that eukaryotes almost exclusively targeted the hydroxyl amino acids serine, threonine, and tyrosine for signal transduction (Kennelly and Potts, 1996; Ullrich and Schlessinger, 1990), while bacteria made use of histidine and the carboxyl amino acids as phosphoacceptors. (Kennelly and Potts, 1996). It was further believed that bacteria were incapable of phosphorylating tyrosine which, in the past, has been suggested to be the primary indicator of signal transduction in multicellular organisms (Ullrich and Schlessinger, 1990). In recent years, evidence to the contrary has surfaced. Tyrosine phosphorylated proteins have been found in both Gram positive and Gram negative organisms (Atkinson *et al.*, 1992; Dadssi and Cozzone, 1990; Duclos *et al.*, 1996; Kennelly and Potts, 1996; South *et al.*, 1994; Waters *et al.*, 1994). Phosphotyrosine is not an abundant phosphoamino acid. Its rarity may indicate that tyrosine phosphorylation is reserved for the regulation of control pathways (Hunter and Cooper, 1985). In *S. griseus*, high concentrations of cAMP are inhibitory to tyrosine phosphorylation and this inhibition is coincident with repression of antibiotic production and morphological differentiation (Kang *et al.*, 1999). Recently, a phosphotyrosine protein phosphatase has been identified in *Streptomyces coelicolor* (Li and Strohl, 1996) indicating that tyrosine phosphorylation may play a role in the development of this organism.

Protein serine/threonine kinases were long thought to be unique for eukaryotic cells until the first serine/threonine kinase gene, *pkn1* was identified in *Myxococcus xanthus* (Munoz-Dorado *et al.*, 1991). This gene was expressed exclusively during development. In 1995, a second *pkn* gene, *pkn2* was identified (Udo *et al.*, 1995). Since the discovery of eukaryotic-like protein serine/threonine kinases in *M. xanthus*, these kinases have also been found in other bacterial species. Eukaryotic-like protein kinase families were identified in *Streptomyces coelicolor* A3(2) (Matsumoto *et al.*, 1994; Urabe and Ogawara, 1995), *Streptomyces granaticolor* (Vomastek *et al.*, 1998), cyanobacterium *Anabaena* PCC 7120 (Zhang, 1993; Zhang, 1996; Zhang *et al.*, 1998; Zhang and Libs, 1998), *Myxococcus xanthus* (Hanlon *et al.*, 1997; Munoz-Dorado *et al.*, 1991; Udo *et al.*, 1995; Zhang, 1996) as well as in human pathogens such as *Yersinia pseudotuberculosis* (Galyov *et al.*, 1993) and *Mycobacterium tuberculosis* (Peirs *et al.*, 1997) and in the thermophilic actinomycete *Thermomonospora curvata* (Janda *et al.*, 1996). With the exception of the plasmid-encoded serine/threonine kinase of *Y. pseudotuberculosis* (Galyov *et al.*, 1993), all the prokaryotes for which protein serine/threonine kinases have been described display multicellular developmental characteristics.

Several protein Ser/Thr kinases have been described in streptomycetes (Table 1.5). AfsK, a membrane-associated kinase has been shown to phosphorylate a global regulatory protein AfsR *in vitro*, while the function of the other four kinases, PkaA and PkaB (Urabe and Ogawara, 1995) and Pkg3 and Pkg4 (Vomastek *et al.*, 1998) are still unknown. Pkg2 from *S. granaticolor* was the first transmembrane protein serine/threonine kinase identified in streptomycetes (Nadvornik *et al.*, 1999). Transmembrane protein serine/threonine kinases have been discovered in *M. xanthus*

(Hanlon *et al.*, 1997; Udo *et al.*, 1995; Zhang *et al.*, 1996) which also undergoes multicellular development suggesting that these kinases may play a role as receptors for developmental signals. It is interesting that sequencing of the *E. coli* genome did not reveal any eukaryotic-like protein serine/threonine kinases (Blattner *et al.*, 1997; Nadvornik *et al.*, 1999). This organism does not differentiate suggesting that perhaps serine/threonine kinases play a role in differentiation of bacteria that exhibit a multicellular mode of growth. The suggestion that this family of proteins may be involved in development in *Streptomyces* is strengthened by the finding that inhibitors of protein serine/threonine and tyrosine kinase such as staurosporine and K-252a inhibit aerial mycelium formation and antibiotic production in *S. coelicolor* A3(2) (Hong and Horinouchi, 1998) and in *S. griseus* (Hong *et al.*, 1993).

It appears that streptomycetes have integrated classical two-component signal transduction pathways with serine/threonine and tyrosine phosphorylation cascades for the regulation of processes involved in biochemical and morphological differentiation. How these signals are communicated from the environment to the substrate mycelium to the aerial hyphae are only starting to be discerned.

I.6 Scope of Thesis

The mechanisms which regulate morphogenesis and antibiotic production have not yet been elucidated in detail. While it is clear that the products of the *bld* genes do exert a regulatory influence, the level of this influence will not be fully understood until the targets of the *bld* genes are identified and characterized. In order to advance our knowledge of the regulatory role that *bldA* plays in the process of differentiation, this research project attempted to identify targets of *bldA* in *Streptomyces coelicolor*. *bldA* targets are TTA-containing genes that require the *bldA*-encoded tRNA for translation of their mRNAs. Since *bldA* acts at the level of translation, attempts were made to identify proteins not expressed in *bldA* mutants by comparing total cellular proteins from *bldA*$^+$ and *bldA*$^-$ strains. However, because of the complexity of the protein profiles, the identification of *bldA* targets, expected to be small in number, proved difficult. Since it is known that reversible protein phosphorylation plays a fundamental role in the regulation of physiological functions of various proteins (Bourret *et al.*, 1991; Parkinson, 1993; Stock *et al.*, 1989) and since there is evidence for a role in regulating secondary metabolism and cell differentiation in *Streptomyces*, differences in *in vitro* phosphorylated protein profiles were assessed to limit the search for *bldA* targets. It should be noted that not all *bldA* targets were addressed utilizing this experimental strategy, since not all *bldA* targets are likely to be phosphorylated.

The research described in the following pages focuses on the investigation of a phosphorylated 32 kDa putative *bldA* target. This protein represents an indirect *bldA* target since its DNA sequence was devoid of a TTA codon yet its phosphorylation status appeared to be *bldA*-dependent. Indirect *bldA* targets can generally be defined as proteins that do not possess a TTA codon(s), but are regulated by a TTA-containing regulator. Knowledge of how this and other *bldA* targets are involved in the regulation of antibiotic production in *Streptomyces* will ultimately aid in strategies aimed at increasing commercial production of industrially important compounds. In addition, investigations of phosphorylated *bldA* targets will contribute to our understanding of the role phosphorylation plays in regulating secondary metabolism and cell differentiation in *Streptomyces*.

II MATERIALS AND METHODS

II.1 Materials

All reagents for two-dimensional gel electrophoresis were purchased from Millipore, Bedford, MA. Protein molecular weight markers were purchased from Bio-Rad, Hercules, CA. Polyvinylidene difluoride (PVDF) Immobilon-P^{SQ} membranes were purchased from Millipore, Bedford, MA. Western blot chemiluminescence reagents were purchased from NEN Life Science Products, Boston, MA. Nitrocellulose membranes, Ponceau S, Phenylmethylsulfonyl Fluoride (PMSF), 2,2-Dihydroxy-1,3-indanedione (Ninhydrin), *O*-phospho-DL-Serine, *O*-phospho-DL-tyrosine, *O*-phospho-DL-threonine, sodium metavanadate, 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), and Pepstatin A were purchased from Sigma-Aldrich, St. Louis, MO. Pefabloc (4-(2-Aminoethyl)-benzenesulfonyl fluoride, hydrochloride) was obtained from Boehringer Mannheim, Indianapolis, IN. β-Mercaptoethanol was purchased from BDH Limited, Poole, UK. *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) was purchased from Calbiochem, La Jolla, CA. Formic acid was obtained from Fisher Scientific, Fairlawn, NJ. Plastic-backed thin layer cellulose chromatography (TLC) sheets without fluorescent indicator were purchased from EM separations Gibbstown, NJ. Constant boiling 6 N Hydrochloric Acid (Sequenal grade) was purchased from Pierce, Rockford, IL. PY72 monoclonal antibody was a gift from Dr. Hanne Ostergaard, University of Alberta, Edmonton, AB. Goat anti-mouse^{HRP} was obtained from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA. DEAE Sepharose FF was purchased from Pharmacia Biotech, Piscataway, NJ.

Restriction endonucleases were obtained from either Boehringer Mannheim, Indianapolis, IN or New England Biolabs Ltd., Mississauga, ON. T4 DNA ligase, Polynucleotide kinase, RNase-free DNase, Klenow fragment of *E. coli* DNA polymerase, and *S1* nuclease were purchased from Boehringer Mannheim, Indianapolis, IN. Avian Myeloblastosis Virus (AMV) reverse transcriptase, *C therm* and Expand DNA polymerase mix (a mixture of Taq and Pwo polymerase) were obtained from Boehringer Mannheim, Indianapolis, IN. RNA Guard was purchased from Pharmacia Biotech, Piscataway, NJ. Deoxyribonucleoside triphosphates were purchased from Boehringer Mannheim, Indianapolis, IN. All enzymes were used according to manufacturers' specifications.

Bacto peptone, yeast extract, Casamino acids, malt extract, tryptone, and Bacto Agar were purchased from Difco Laboratories, Detroit, MI. Trypticase soy broth (TSB) was purchased from BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD. Amino acids were obtained from Sigma-Aldrich, St. Louis, MO. Isopropyl β-D-thiogalactopyranoside (IPTG) was obtained from Boehringer Mannheim and 5- bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) was obtained from American BiOrganics, Niagara Falls, NY.

Ampicillin, and kanamycin were obtained from Sigma-Aldrich St. Louis, MO. Thiostrepton was a gift from S. Lucania, Bristol-Myers Squibb, Princeton, NJ. Apramycin was obtained from E. Seno, Eli Lilly and Company, Indianapolis, IN. Apralan was obtained from Provel, a division of Eli Lilly Canada Inc., Scarborough, ON.

Agarose was obtained from ICN Biochemicals, Inc., Aurora, OH. Ammonium persulfate (APS) and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were obtained from Gibco BRL (Bethesda Research Laboratories), Gaithersbury, MD. Premixed 40% polyacrylamide solutions (29:1, acrylamide:N,N'-methylene bisacrylamide; 19:1, acrylamide:N,N'-methylene bisacrylamide) were obtained from Fisher Scientific, Fairlawn, NJ. Polyethyleneglycol (PEG) 1000 MW was obtained from NBS Biologicals, Hatfield, UK. Lysozyme, dithiothreitol (DTT), diethyl pyrocarbonate (DEPC) and spermidine, were obtained from Sigma-Aldrich, St. Louis, MO. Bovine serum albumin (Fraction V) was obtained from Boehringer Mannheim, Indianapolis, IN.

Radioactively labelled [γ-^{32}P] ATP was obtained from ICN Biochemicals, Irvine, CA. [α-^{32}P] dCTP was obtained from Amersham, Arlington Heights, IL. Thermo Sequenase™ radiolabelled terminator cycle sequencing kit was obtained from USB Corporation, Cleveland, OH. Nuc-Trap® probe purification columns were obtained from Stratagene, La Jolla, CA. Micro Bio-Spin® chromatography columns were obtained from Bio-Rad, Hercules, CA. Oligonucleotide primers were obtained from the Department of Biological Sciences DNA Synthesis Laboratory, University of Alberta, Edmonton, AB. The sequences of the oligonucleotide primers used in this study are listed in Table II.1.1.

All other chemicals used in this study were reagent grade.

DNA sequences and putative protein sequences were analyzed with DNA Strider, a software program designed and written by C. Marck (Commissariat a l'Energie Atomique, France). FRAME, a program written by Bibb *et al.*, (1984) and adapted for the Apple MacIntosh by C. Jensen (or alternatively online at www.nih.go.jp/~jun/cgi-bin/frameplot.pl) was used to examine open reading frames (ORFs). Protein and

Table II.1.1. Oligonucleotide Primer Sequences

Primer	Sequence (5' → 3')	Region of Homology/Feature	Use
Universal	GTAAAACGACGGCCAGT	Upstream of MCS of pUC and M13 based vectors	Sequencing
BKL 54	CCGCCTTCGCCACCGGT	Complementary to *Streptomyces* 16S rRNA transcripts	Control for RNA loading in Northern analysis
DBG6	GCCAAGAAGAACCACCGCCAAG	Internal to *hrdB* of *Streptomyces* at 5' end	For two-step RT-PCR
DBG7	GACCTTGCCGATCTGCTTGAG	Internal to *hrdB* of *Streptomyces* at 3' end	For two-step RT-PCR
JLS 2	ATGGG(AG)CACGC(CG)GG(CG)GC(GC)ATCGT	Internal peptide sequence of 32 kDa protein (MGHAGAIV)	Probe for Southerns
JLS 3	GG(CG)GAGGT(CG)GG(GC)AACGC(CG)GAGCAG	Internal peptide sequence of 32 kDa protein (GEVGNAEQ)	Probe for Southerns
JLS 10	TACCCCGTGCG(GC)CC(GC)CG(GC)TAGCA	Complementary sequence to JLS2	For attempted PCR of gene for 32 kDa protein
JLS 11	CC(GC)CTCCA(CG)CC(CG)TTGCG(GC)CTCGTC	Complementary sequence to JLS3	For attempted PCR of gene for 32 kDa protein
JLS 16	GC(GC)AC(GC)GT(GC)GG(GC)GAGGT(GC)GG(GC)AA CGC(GC)GAGCAGAGCAACATCAACTACGG	Longer version of JLS3	Probe for Southerns
JLS 17	GC(GC)AC(GC)GT(GC)GG(GC)GAGGT(GC)GG(GC)AA CGC(GC)GAGCAGTC(GC)AACATCAACTACGG	Longer version of JLS3	Probe for Southerns
JLS 34	GG(GC)GTCTT(GC)CC(GC)AC(TC)TT(AGCT)AC	Based on homology to *M.tuberculosis* SCS alpha chain corresponds to VKVGKTP (amino acids 282 to 288)	For PCR amplification of SCS from *Streptomyces coelicolor*
JLS 35	GACAACAAGGT(ACGT)AT(ACT)GT(ACGT)CA	Based on homology to *M. tuberculosis* SCS alpha chain corresponds to DNKVIVQ (amino acids 11 to 17)	For PCR of SCS from *S. coelicolor*
JLS 36	ACCCC(GC)TTCGG(TGCA)CT(TC)CC(TGCA)GC	Based on homology to *M. leprae* 50S ribosomal protein L2 corresponds to WGKPEGR	For PCR of 50S ribosomal protein L2
JLS 37	AAGCC(CG)GAGAA(AG)GC(AGCT)(CT)T(AGCT)ATG	Based on homology to *M. leprae* 50S ribosomal protein L2 corresponds to KPEKALM	PCR amplification of 50S ribosomal protein L2
JLS 46	GGCTAAGCTTGTCACGGTCGTCTTCGT	229 bp from start of *sucD*	For internal PCR of *sucD* with *HindIII* site
JLS 47	CCACTCTAGACATCGTCCGGCCCTCG	738 bp from start of *sucD*	For internal PCR of *sucD* with *XbaI* site
JLS48	CCCAGAGGTTGAGGACG	1054 bp from start of *sucD*	For 3' PCR of *sucD* for sequence determination
JLS 49	CAACTCATGTACGAACTGC	506 bp from start of *sucD*	For 3' PCR of *sucD* for sequence determination
JLS 50	GTGGAGAAGCCGATGTC	523 bp from start of *sucD*	For 5' PCR of *sucD* for sequence determination
JLS 51	ACAGGCCGGATAAGGAGA	26 bp upstream of translation start of *sucD*	For 5' PCR of *sucD* for sequence determination
JLS 52	TGGTGAGGTAGATCGCCAT	18 bp from start of *sucD* coding region	For primer extension of *sucD*
JLS53	TCCCGTGTTCCTTGAAGA	48 bp downstream of *sucC* coding region	For S1 Mapping of *sucC*
JLS54	GTGTGCTTCATGCCCTCG	62 bp downstream of translation start of *sucD*	For S1 mapping *sucD*
JLS55	GCGCAAGCTTACGCAGCCGTCGTAGATG	272 bp upstream of the translation start of *sucC*	For S1 mapping of *sucC*
JLS56	GCGCAAGCTTCTCACCAAACCGCTCGTC	185 bp upstream of *sucD* coding region	S1 mapping of *sucD*
JLS57	CAGGTCCATCTCGGCTCC	Spans the start of the translation start of *sucC* coding region	S1 mapping and primer extension of *sucC*

nucleotide similarities were determined through the use of the Basic Local Alignment Search Tool (BLAST; Altschul *et al*., 1990). Protein and nucleic acid sequence alignments, as well as contig analysis, were performed using Lasergene software by DNAStar, Madison, WI.

II.2 Bacterial Strains, Plasmids and Phages

II.2.1 Bacterial Strains, Plasmids and Phages

The *Streptomyces* and *Escherichia coli* strains used for this study are listed in Table II.2.1.1. The plasmids and phages used are listed in Table II.2.1.2. Plasmids generated in this study are listed in Table II.2.1.3.

II.2.2 Maintenance of *Streptomyces* Strains

Sporulating *Streptomyces* strains were maintained as frozen glycerol spore stocks. *Streptomyces coelicolor* and *Streptomyces lividans* strains were grown on R2YE agar (Hopwood *et al*., 1985) at 30°C. Auxotrophic strains were grown on media supplemented with Tiger's Milk (10 mg/mL L-arginine, 7.50 mg/mL each of L-cystine, L-histidine, DL-homoserine, L-leucine, L-phenylalanine, L-proline, 1.50 mg/mL of adenine and uracil and 0.1 mg/ml nicotinamide). For the preparation of spore stocks, sporulating plate cultures were flooded with sterile Milli-Q water and spores were gently scraped from plates with a sterile spatula. To disperse the spores, the spore suspension was placed in a Braunsonic 220 water-bath sonicator then filtered through sterile, non-absorbent cotton wool packed into a 15 mL conical centrifuge tube with a small hole in

Table II.2.1.1 Bacterial Strains

Strain	Genotype	Reference or Source
***Streptomyces coelicolor* A3(2)**		
J1501	*hisA1, uraA1, strA1, pgl,* SCP1^{-}, SCP2^{-}	John Innes Institute; Chater, *et al.*, 1982
J1681	Δ*bldA, hisA1, uraA1, strA1, pgl,* SCP1^{-}, SCP2^{-}	John Innes Institute; Leskiw *et al.*, 1993
***Streptomyces lividans* 66**		
1326	SLP2, SLP3	John Innes Institute; Lomavskaya *et al.*, 1980
TK24	*Str-6,* SLP2^{-}, SLP3^{-}	John Innes Institute; Hopwood *et al.*, 1983
Escherichia coli		
DH5α	F^{-}, *ϕ80dlacZ* ΔM15, Δ(*lacZYA-argF*)*U169, deoR, recA1, endA1, hsdR17* (r_K-, m_{K+}), *phoA, supE44,* λ^{-}, *thi-1, gyrA96, relA1*	GIBCO-BRL Life Technologies, Inc., Gaithersburg, MD.
ET12567	F^{-} *dam13::Tn9, dcm6, hsdM, hsdR, RecF143, zjj202::TN10, galK2, galT22, ara14, lacY1, xyl5, leuB6, thi1, tonA31, rpsL136, hisG4, tsx78, mtl1, glnV44*	Gift from D. MacNeil; Merck Sharp & Dohme Research Laboratories; MacNeil *et al.*, 1992

Table II.2.1.2 *Streptomyces* and *Escherichia coli* Plasmids and Phages

Plasmid or Phage	Relevant Characteristic or Genotype	Reference or Source
***Streptomyces* plasmids**		
pIJ486	High copy number cloning vector (*tsr*) Contains *Streptomyces* pIJ101 replicon	John Innes Institute; Ward *et al.*, 1986
pIJ584	Derivative of pIJ486; contains the *bldA* gene	Leskiw *et al.*, 1993
pIJ4083	Contains the promoterless catechol 2,3 dioxygenase gene (*xylE*); multicopy; Tsr^R	John Innes Institute; Clayton and Bibb, 1990
pIJ4083/*ermE**	*xylE* under control of the mutant form of the promoter for the erythromycin resistance-encoding gene	Petrich *et al.*, 1992
***E. coli* plasmids**		
pUC119	High copy number phagemid cloning vector (Amp^R)	J. Vieira, Waksman Institute of Microbiology, Rutgers University, Piscataway, NJ; Vieira and Messing, 1987
pUC119 + Ap^R	Ap^R cassette in *Xba*I-*Eco*RI site of MCS of pUC119	S.E. Jensen, Personal Communication
pIJ2925	pUC18 derivative with polylinker flanked by *Bgl*II sites	Janssen and Bibb, 1993
pAU5	pIJ2925 with *tsr* marker	Geibelhaus *et al.*, 1996
pSET152	*E. coli*-*Streptomyces* shuttle vector; integrates into ϕC31 *att* site (Apr^R)	Northern Regional Research Center, Peoria, IL; Bierman *et al.*, 1992
pK184/pK194	Km^R, *lacZα*, ColE1 replicon	Jobling and Holmes, 1990
pBluescript SK+/KS+	General cloning vector; phagemid; Amp^R	Stratagene
Phages		
KC860	ϕC31::*xylE* vector; allows insert-directed activation of *xylE*	John Innes Institute; Bruton *et al.*, 1991
KC900	KC860 containing *actI*::*xylE* fusion	John Innes Institute; Bruton *et al.*, 1991

Table II.2.1.3. Plasmids Generated in this Study.

Plasmid	Parent Vector	Features/Comments	*S. coelicolor* Cosmid
pA4-66	pBluescript KS+	*Sst*II fragment (1.7 kb) SCI7.02c†- possible oxidoreductase, 281 aa (75% GC)	SCI7
p56-19	pUC119	*Sal*I fragment (~2 kb) *SC4G2.14 - possible ATP-dependent helicase, 731 aa (68% GC)	SCG42
p59-41	pUC119	*Xma*I fragment (1.1 kb) *SC5H1.18 - possible membrane protein, function unknown, 400 aa (76% GC) *SC5H1.19c - hypothetical protein, 136 aa, contains TTA codon (possible target for *bldA* regulation) (72% GC)	SC5H1
p57-38	pUC119	*Sal*I fragment (~2 kb) DesR homolog (similarity to *S. lividans*) (71% GC)	2St10A7
p45-37	pUC119	*Xma*I fragment (1.1 kb) SC5F2A.04 - possible integral membrane protein, 302 aa (74% GC) *SC5F2A.05 - possible regulator protein, 138 aa, homologous to *ssgA* protein of *S. griseus* (72% GC)	SC5F2A
p64-17 p64-44 p64-25	pUC119	*Xma*I fragment (1.1 kb) *SCF11.09c - possible oxidoreductase, 508 aa (73% GC) *SCF11.10c - hypothetical transcriptional regulator, 281 aa (72% GC)	SCF11
p59-17	pUC119	*Sal*I fragment (~2 kb) SC5H1.27 - hypothetical protein, function unknown, 199 aa (70% GC) SC5H1.28c - possible oxidoreductase, 305 aa (74% GC)	SC5H1
p33-27	pBluescript KS+	*Sal*I fragment (~2 kb) *SCE134.08 - possible integral membrane efflux protein, 500 aa (70% GC) SCE134.09 - possible maturase-related protein, 145 aa (68% GC) SCE134.10 - hypothetical protein, function unknown, 320 aa (69% GC) *SCE134.11c - possible DNA methylase, 248 aa (69% GC)	SCE134
pGD1-6	pUC119 + Ap^{R}	Contains an internal 527 bp *sucD* fragment; gene disruption construct, Ap^{R}	N/A
pGD7-18	pAU5	Contains an internal 527 bp *sucD* fragment; gene disruption construct, Tsr	N/A
pJLS4565CP	pK184	Contains a 4565 bp *Cla*I/*Pvu*I fragment containing coding sequence for SC8A6.05, SCS, and part of SC8A6.08, cloned into *Sph*I, Km^{R}	N/A

*Partial sequence on plasmid. N/A-Not applicable. †'SC' designations refer to *S. coelicolor* cosmids. Open reading frame (ORF) designations follow 'SC' the bottom. The filtered suspension was centrifuged for 10 minutes at 2,900 rpm in a PR-J centrifuge, and the pellet was resuspended in glycerol to a final concentration of 20% (v/v), dispensed into sterile 5 mL Bijou bottles and stored at -20°C.

Bald (non-sporulating) mutant strains were maintained as frozen mycelial stocks prepared by cultivating cultures on sterile, cellophane discs applied to the surface of R2YE agar plates which prevent penetration of substrate mycelia into the agar. Mycelia were scraped from plates with a sterile spatula, suspended in sterile Milli-Q water, homogenized, centrifuged, resuspended to a final concentration of 20% (v/v) glycerol, and stored at -20°C.

Plasmid containing strains bearing thiostrepton (tsr) or apramycin (apr) resistance determinants were propagated on media containing 50 µg/mL of the appropriate antibiotic.

II.2.3 Maintenance of *Escherichia coli* Strains

E. coli strains were maintained as frozen glycerol stocks. Sterile glycerol was added to 3-5 mL overnight LB cultures to a final concentration of 20% (v/v) glycerol. Cultures of strains containing plasmids were supplemented with 100 µg/mL ampicillin, 100 µg/mL kanamycin or 50 µg/mL Apralan. Glycerol stocks were flash frozen in a dry ice-ethanol bath and stored at -70°C.

II.2.4 Maintenance of Phages

Phages used in this study were maintained as single plaque soak outs in aliquots of 1.5 mL DNB (Difco nutrient broth) at 4°C (see below).

II.3 Protein Analysis

II.3.1 Liquid Culture

II.3.1.1 Culture Conditions for Cell-Free Extract Preparation

For the preparation of liquid culture cell-free extracts, *S. coelicolor* was cultivated at 30°C on a platform shaker at 250 rpm in duplicate 25 mL cultures in 250 mL spring flasks containing YEME (yeast extract, malt extract) medium (Hopwood *et al.*, 1985) supplemented with 5 mM $MgCl_2$, 0.5% glycine and 0.75% Tiger Milk (Super YEME).

II.3.1.2 Preparation of Cell-Free Extracts

To prepare liquid culture cell-free extracts, cultures grown in Super YEME were harvested at 24, 30, 36, 42, and 48 hours by centrifugation in sterile Universal bottles in a PR-J centrifuge at 4°C. Before centrifugation, the cultures were diluted with chilled, sterile 0.1 M HEPES buffer as necessary to decrease the sucrose concentration and help pellet the mycelia. Pellets were washed with cold 0.1 M HEPES buffer and transferred to centrifuge tubes. The mycelia were pelleted again and then resuspended in lysing buffer (0.1 M HEPES, 1 mM Pefabloc, 1 mM PMSF, 1 µM Pepstatin A, and 0.5 mg/mL lysozyme) and incubated at 37°C for 10 minutes. The partially lysed mycelia were then sonicated on ice five times for 15 seconds with cooling intervals of 15 seconds at 40 watts with a Braunsonic sonicator. The lysate was then centrifuged 10 minutes at 4°C at 10,000 x g to remove cell debris. Aliquots of the cell-free extract were flash-frozen in a dry ice-ethanol bath and stored at -70°C.

II.3.2 Surface Culture

II.3.2.1 Surface Culture Conditions for Cell-Free Extract Preparation

For the preparation of surface culture cell-free extracts, R2YE agar supplemented with Tiger's Milk was used as the growth medium. After pouring, plates were dried under laminar flow for one hour. Sterile cellophane disks were placed on the agar surface and *S. coelicolor* spores or mycelia were plated on the surface of these disks. The cultures were incubated at 30°C.

II.3.2.2 Preparation of Cell-Free Extracts

Surface culture cell-free extracts were prepared by harvesting cultures at 15, 24 or 48 hours by scraping the mycelia from the cellophane disks with a sterile spatula into centrifuge tubes. The mycelia were then suspended in lysing buffer and incubated for 10 minutes at 37°C and sonicated on ice five times for 15 seconds with cooling intervals of 15 seconds at 40 watts with a Braunsonic sonicator. The lysate was then centrifuged 10 minutes at 4°C at 10,000 x g to remove cell debris. Aliquots of the cell-free extract were flash-frozen in a dry ice-ethanol bath and stored at -70°C.

II.3.3 Protein Quantification

Protein was assayed by the method of Bradford (1976) using the Bio-Rad dye reagent according to the microassay procedure described by the supplier. Bovine gamma globulin was used as the protein standard.

II.3.4 One-Dimensional Gel Electrophoresis

Protein samples were subjected to electrophoresis on 10%, 12.5% or 15% polyacrylamide gels containing 0.1% SDS using the gel and buffer system described by Laemmli (1970). Unstained, Bio-Rad broad range molecular weight marker proteins included cytochrome *c* (12,500), chymotrypsinogen A (25,000), ovalbumin (45,000), and bovine serum albumin (67,000). Bio-Rad Kaleidoscope prestained standards consisted of myosin (~203,00), β-galactosidase (~126,000), bovine serum albumin (~71,000), carbonic anhydrase (~41,800), soybean trypsin inhibitor (~32, 800), lysozyme (~17,900), and aprotinin (~7,500) and were supplied calibrated for each batch since the binding of the dyes is an uncontrolled reaction and produces lot-to-lot variation in the molecular weights. Bio-Rad pre-stained molecular weight markers were also supplied calibrated per batch.

II.3.5 Two-Dimensional Gel Electrophoresis

All glassware used for two-dimensional gel electrophoresis was acid washed in chromic acid cleaning solution and rinsed thoroughly first with distilled water, then with Milli-Q water. The Millipore Investigator 2-D electrophoresis protocol was adapted for these experiments.

II.3.5.1 Isoelectric Focusing Gels

Isoelectric focusing gels were cast by placing IEF tubes (1.5 mm ID, 6.0 mm OD, 180 mM) in 13 x 100 mm test tubes in a Bio-Rad Model 225 tube gel casting stand. IEF tubes were marked 14 cm from one end with a pen. The IEF tube was then connected to a 1 mL Tuberculin syringe using a small piece of Tygon tubing (3/16" ID x 1/4" OD and ~2 cm in length). The IEF/syringe assembly was placed into a test tube in the casting stand. Aliquots of IEF gel solution (9.5 M urea, 2% (w/v) Nonidet P-40, 4.1% acrylamide (29:1), 5 mM CHAPS; stored at -70°C) were thawed at 30°C and degassed for 15 minutes. Millipore 3-10/2D Ampholyte (350 µL) was added and the solution gently mixed by inversion to minimize foaming. Ammonium persulfate (APS, 10 µL) was added to the vial to initiate polymerization and the solution was again mixed gently by inversion. A 0.5 mL aliquot of the acrylamide solution was dispensed into each test tube. The syringe assembly was used to pull the liquid into each IEF tube to the 14 cm mark. The IEF capillary tubes were allowed to sit undisturbed with the syringes attached for 2 hours at room temperature to allow complete polymerization.

The IEF gels were prefocused in the following manner: The IEF gel tubes were placed in grommets with the line marking 14 cm below the bottom of the assembly in the Bio-Rad IEF apparatus. The capillary tubes and top reservoir were filled with cathode buffer (0.1 N NaOH; degassed) while the bottom reservoir was filled with two litres of anode buffer (100 mM phosphoric acid; degassed). Overlay buffer (5 µL; 0.5 M urea, 0.2% (v/v) Nonidet P-40, 0.1% (v/v) Ampholytes (40% stock), 50 mM DTT) was added to each IEF tube gel with a Hamilton syringe. The voltage was set at 1000 volts and 0.5 mA (constant current). Prefocusing was complete when the voltage reached 1000 volts.

To prefocused IEF tube gels, 10 µL of each sample (which had been digested 45 minutes with 1 mg/mL DNase I and 0.25 mg/mL RNase A) in sample buffer composed of 0.5 M urea, 2% Triton X-100, 5% β-mercaptoethanol, 1.6% Biolyte 5/7 ampholyte, 0.4% Biolyte 3/10 ampholyte was applied with a Hamilton syringe. Samples were kept at 30°C prior to loading to prevent precipitation of urea. The samples were run for 19 hours at 1000 volts.

Once the focusing of the IEF gels was complete, the tubes were rimmed top and bottom with Milli-Q water using a gel extrusion needle. The acidic end of the tube gel was marked with a drop of Bromophenol blue dye. The tube gels were extruded directly onto a labelled strip of Parafilm. The tube gels were equilibrated in IEF Equilibration Buffer (0.3 M Tris base, 0.075 M Tris-HCl, 3% SDS, 50 mM DTT, 0.01% Bromophenol blue) for 15 minutes.

II.3.5.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (Second Dimension)

Degassed sodium dodecyl sulfate polyacrylamide gels (12.5%) were used for resolution in the second dimension. Prior to loading the IEF tube gels onto the second dimension gel, excess equilibration buffer was removed. The tube gel was loaded onto the slab gel by angling the parafilm strip between the two gel plates; a spatula was used to gently guide the tube gel so that there were no air bubbles between the two gels. Gels were run at 40 mA/gel using the gel and buffer system described by Laemmli (1970). Once gel electrophoresis in the second dimension was complete, the basic end of the gel was notched for orientation purposes. Gels to be stained with either silver or Coomassie brilliant blue were fixed overnight in 50% methanol:10% acetic acid while gels to be autoradiographed were transferred to either nitrocellulose or PVDF membrane.

II.3.6 Protein Phosphorylation

II.3.6.1 *In vitro* Protein Phosphorylation Assays

Two methods were employed for *in vitro* phosphorylation studies. The first method used was described by Wang and Koshland (1981). The assay mixture, in a 50 µl reaction volume consisted of 10 mM Tris (pH 7.5), 1 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 1 μM [γ-^{32}P] ATP and 100-200 μg of protein. Reactions were incubated at 37°C for 5 minutes and quenched by the addition of 25 μL 3X Laemmli's final sample buffer (0.0625 M Tris-HCl; pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.001% Bromophenol blue). Samples were denatured at 90°C for 5 minutes prior to analysis on SDS-polyacrylamide gels. The second method used for *in vitro* protein phosphorylation of cell-free extracts was performed as described by Hong *et al.* (1993). The assay cocktail consisted of 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 μM [γ-^{32}P] ATP, and 10-100 μg protein. The reactions were incubated at room temperature for 5 minutes. Reactions were quenched by the addition of 0.5 volume of 3X Laemmli's final sample buffer. Samples were heat denatured at 90°C for 2-5 minutes and then analyzed by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes or to PVDF membranes and autoradiographed either by exposure to Kodak X-OMAT AR film or by exposure to a phosphorimager screen. X-ray film was developed using a FUJI RGII X-ray Film Processor while the phosphorimager screen was scanned using a Molecular Dynamics Model 445 S1 Phosphorimager.

II.3.6.2 Optimization of Mn^{2+} and Ca^{2+} Concentrations in Phosphorylation Reactions

In order to determine the effect of Mn^{2+} on the phosphorylation profile, increasing $MnCl_2$ concentrations were added to the phosphorylation cocktail ranging from 5 mM to 50 mM. The rest of the labelling procedure remained unchanged.

To determine the effect of Ca^{2+} on the phosphorylation profile, increasing $CaCl_2$ concentrations ranging from 5 mM to 50 mM were added to the phosphorylation cocktail. The rest of the labelling procedure remained unchanged.

II.3.7 Transfer of Protein to Nitrocellulose

Following separation of proteins by electrophoresis, gels were equilibrated in transfer buffer (25 mM Tris-HCl/pH 8, 192 mM glycine, 0.1% SDS, 20% methanol) for 30 minutes with gentle shaking. The nitrocellulose membrane was prewetted in Milli-Q water and then equilibrated in transfer buffer for 15 minutes. The transfer cassette of the Bio-Rad Transblot apparatus was assembled according to the manufacturer's instructions. Transfer of proteins to the membrane was allowed to proceed at 60 volts for 5 hours at 4°C.

II.3.8 Transfer of Protein to PVDF

For the transfer of proteins separated by gel electrophoresis to polyvinylidene difluoride (PVDF) membranes, the membrane was cut to size and prewetted by immersion in methanol, rinsed with Milli-Q water and equilibrated in transfer buffer (10 mM CAPS, 10% methanol) for 15 minutes. Protein gels were equilibrated for 15 minutes in CAPS transfer buffer prior to transfer. Transfer of proteins to the PVDF membrane was allowed to proceed at 60 volts for 5 hours at 4°C using a Bio-Rad Transblot apparatus. After transfer was complete, the membrane was washed three times for 15 minutes to remove residual methanol.

II.3.9 Protein Staining

II.3.9.1 Silver Staining

Two methods of silver staining proteins separated by SDS-polyacrylamide gel electrophoresis were used. The first method (Bloom *et al.*, 1987; Merril *et al.*, 1984), was carried out as follows: the gel was soaked for 10 minutes in formaldehyde fixing solution (40% methanol, 0.5 mL 37% formaldehyde/litre), washed twice for five minutes in Milli-Q water, then soaked for 1 minute in 50 mL 0.2 g/L $Na_2S_2O_3$ then washed with Milli-Q water twice for 20 seconds per wash. The gel was then soaked for 10 minutes with 50 mL 0.1% $AgNO_3$ then washed briefly with water followed by development in 50 mL freshly prepared thiosulfate developing solution (3% (w/v) sodium carbonate, 0.0004% (w/v) sodium thiosulfate, 0.5 mL of 37% formaldehyde) until desired band intensity was reached. Development was stopped by the addition of 5 mL 2.3 M citric acid/100 mL thiosulfate developing solution for ten minutes. The second silver stain method used was as described by Morrissey (1981). After gels were fixed two hours to overnight in 50% methanol, 10% acetic acid, they were washed twice in 200 mL Milli-Q water followed by reduction in 250 mL of DTT (5 μg/mL) solution for 30 minutes. Gels were soaked in 200 mL of 0.1% silver nitrate solution for 30 minutes. Gels were then washed with 100 mL Milli-Q water for 2 minutes, 200 mL Milli-Q water for 5 minutes, and 150 mL Milli-Q water for 5 minutes. Development was achieved by the addition of 200 mL developer (0.28 M Na_2CO_3, 0.6% (v/v) of 37% formaldehyde). Soaking the gel in 10% acetic acid stopped development. Stained gels (of either method) were then washed for 10 minutes in Milli-Q water, soaked in gel drying solution (10% (v/v) ethanol, 4% (v/v) glycerol) for at least 45 minutes. For preservation, gels were dried using the BioGel Wrap™ gel drying system (BioDesign, Inc., Carmel, NY).

II.3.9.2 Coomassie Brilliant Blue Staining

To visualize proteins resolved on SDS-polyacrylamide gels by Coomassie Brilliant blue staining, gels were soaked with three volumes of fixative (50% methanol, 10% acetic acid) for 2 hours and stained with Coomassie staining solution (10% glacial acetic acid, 50% methanol, 0.2% (w/v) Coomassie Brilliant Blue (R-250)) for 4 hours to overnight. Gels were destained in 50% methanol, 10% acetic acid for two hours changing destain solution as required (Merril, 1990). The addition of Kimwipes, which absorbed the Coomassie blue dye, aided in the destaining process.

II.3.9.3 India Ink Staining

Nitrocellulose or PVDF membranes to be stained were placed in a plastic dish on an orbital shaker and washed with Tween 20 solution (0.3% Tween 20 in phosphate buffered saline (PBS; pH 7.4)) three times for 30 minutes each at 37°C followed by two additional washes of 30 minutes at room temperature. The prewashed membranes were stained for 3 hours to overnight in India ink solution (0.1% Pelikan 17 in Tween 20 Solution). To destain, membranes were rinsed in Tween 20 solution until protein bands appeared black against a gray background (Hancock and Tsang, 1983).

II.3.9.4 Ponceau S Staining

Nitrocellulose membranes containing protein were immersed in Ponceau S solution for 10 minutes. Destaining was achieved by rinsing in 5% acetic acid. Protein bands appeared as light red against a pink background.

II.3.10 Western Analysis

II.3.10.1 Electrophoretic Transfer of Proteins to Immobilon

Electrophoretic transfer of proteins was accomplished as follows: PVDF membrane was cut to size and prewetted by immersion in methanol, rinsed with Milli-Q water and equilibrated in transfer buffer (10 mM CAPS, 10% methanol) for 15 minutes.

Protein gels were equilibrated for 15 minutes in CAPS transfer buffer prior to transfer. Transfer of proteins to the PVDF membrane was allowed to proceed at 60 volts for 5 hours at 4°C using a Bio-Rad Transblot apparatus. After transfer was complete, the membrane was washed three times for 15 minutes to remove methanol.

II.3.10.2 Detection of Phosphotyrosine with Anti-Phosphotyrosine Antibodies

PVDF membranes containing protein were blocked for 1 hour to overnight in 5% bovine serum albumin (BSA) in TN buffer (10 mM Tris-Cl; pH 7.4, 0.15 M NaCl; pH 7.6). The primary, monoclonal antibody, PY72, was added at a concentration of 1 µg/mL and incubated for 60 minutes. The membrane was washed with TN buffer briefly twice, washed twice for 5 minutes, followed by one wash for 15 minutes. The second antibody, goat anti-mouse^{HRP} was diluted 1:18,000 in Blocking buffer (TN buffer containing 0.1% Tween 20 and 5% BSA) and incubated with the membrane for 60 minutes. The membrane was then washed in TN buffer containing 0.1% Tween 20 for two quick washes, 4 washes of 5 minutes each and one wash of 15 minutes (Dr. H. Ostergaard, personal communication). Excess buffer was drained, the membrane transferred to a clean glass dish, and ECL (enhanced chemiluminescence) detection components (Renaissance™ Western Blot Chemiluminescence Reagent Plus, Dupont-NEN Life Science) were added in equivalent amounts as recommended by the supplier. The membrane was agitated in this solution for 1 minute, excess detection solution was drained, the membrane was wrapped in Saran wrap and exposed to ECL film (Dupont-NEN RENAISSANCE™ blue film) for 2-30 minutes.

II.3.10.3 Blocking Experiments

Protein samples of interest were run in replicate samples on 10% SDS-PAGE and transferred to PVDF membranes. Membranes containing protein were soaked overnight in 5% BSA in TN buffer containing 0.1% Tween 20. Each phosphoamino acid standard (P-Serine, P-Threonine, P-Tyrosine each at 100 mM) was diluted 1:5 in TN buffer. Each diluted phophoamino acid standard was added separately to 15 mL 2% BSA in TN buffer containing 0.001 mg/mL PY72 and incubated for 30 minutes. This solution was added to a replicate membrane and incubated for 1 hour. The membrane was then washed in TN buffer containing 0.2% Tween 20 for two quick washes, 2 washes at 5 minutes and one wash for 15 minutes. Goat anti-mouse^{HRP} was diluted 1:18,000 in 4% BSA in TBS containing 0.1% Tween 20 and added to the membrane and incubated for 60 minutes. The membranes were washed using 2 quick rinses, 4 washes of 5 minutes, and one wash of 15 minutes in TN buffer containing 0.1% Tween 20 (Dr. H. Ostergaard, personal communication). Detection was as described above.

II.3.10.4 Alkali Enhancement of P-Tyrosine and P-Threonine

Radiolabelled proteins were electrophoresed on 10% SDS-PAGE and transferred electrophoretically to a PVDF membrane. The enhancement of phosphotyrosine and phosphothreonine was carried out as described by Cooper and Hunter (1981) with modifications. After transfer, the membrane was thoroughly rinsed with Milli-Q water to remove residual buffer and detergent. The membrane was incubated 120 minutes at 55°C in 500 mL 1 M KOH in a covered dish. After incubation, the membrane was washed for 5 minutes in 500 mL TN buffer, 5 minutes in 1 M Tris-Cl (pH 7.0), and twice for 5 minutes in 500 mL Milli-Q water. The membrane was then wrapped in Saran wrap and exposed either to Kodak X-OMAT AR X-ray film with an intensifying screen or to a phosphorimager screen and visualized as described previously.

II.3.11 Phosphoamino Acid Analysis

II.3.11.1 Sample Preparation

Proteins were radiolabelled and electrophoresed on 10% or 15% SDS-polyacrylamide gels and then transferred electrophoretically to a PVDF membrane. The membrane was washed several times with Milli-Q water to remove traces of buffer and detergents. The band of interest was located by staining the filter 5-10 minutes in 50 mL India ink solution (1 µl/mL India ink in Tris-buffered saline (TBS), 0.2% (v/v) Tween 20; pH 6.5) and by autoradiography. The piece of membrane containing the band of interest was excised with a clean razor blade, rewet in methanol for 1 minute and then rinsed in Milli-Q water and placed in a screw-capped microcentrifuge tube containing enough 6 M

HCl (constant boiling) to submerge the membrane. The tube was incubated tightly capped in a 110°C oven for 60 minutes, and then cooled on ice. The tube was microcentrifuged for 2 minutes, the liquid hydrolysate was transferred to a fresh microcentrifuge tube and dried using a Speedvac evaporator. The dessicated sample was dissolved in 6 µL Milli-Q water and microcentrifuged at maximum speed for 5 minutes to pellet particulates that would interfere with sample loading.

II.3.11.2 Preparation of Phosphoamino Acid Standards Mixture

A standard solution of phosphoserine, phosphotyrosine and phosphothreonine was prepared in water at a final concentration of 0.3 µg/mL each and stored at –20°C.

II.3.11.3 First Dimension Thin-Layer Electrophoresis

Four origins (corresponding to 3 cm, 3 cm; 3 cm, 8 cm; 8 cm, 3 cm; 8 cm, 8 cm) were labelled in pencil on plastic-backed cellulose thin-layer chromatography (TLC) plates (20 cm x 20 cm x 100 µm). Fifty percent of a given sample was applied to one origin in 0.25-µL aliquots using a Fisherbrand micropipette. The sample spot was dried between each application using a stream of air delivered through a syringe with a 0.45-µm filter attached. One microlitre of non-radioactive phosphoamino acid standards mixture (containing phosphotyrosine, phosphothreonine and phosphoserine) was applied on top of each sample in 0.25-μL aliquots as described above. A blotter composed of two 25 x 25-cm layers of Whatman 3MM paper stapled together at the edges with four 2-cm holes aligned with the origins on the TLC plate was dampened in pH 1.9 electrophoresis buffer (0.58 M 88% formic acid, 1.36 M glacial acetic acid). The wet blotter was placed on the prespotted plate with the origins on the plate in the centres of the four holes in the blotter to achieve both even wetting of the cellulose and concentration of the samples. Once the TLC plate was uniformly wet, the blotter was removed and then placed in the Shandon High Voltage Electrophoresis Model L24 apparatus. Wicks made of Whatman 3MM paper were positioned to overlap approximately 0.5 cm on the right and left sides of the plate. The cover was closed and cold water was circulated through the system and electrophoresed for 25 minutes at 1.5 kV in pH 1.9 buffer. Following electrophoresis, the plate was dried under a stream of cold air for 20 minutes (Boyle *et al.*, 1991; Duclos *et al.*, 1991; Kamps, 1991).

II.3.11.4 Second Dimension Thin-Layer Electrophoresis

Three small blotters (4 x 25-cm, 5 x 25-cm, and 10 x 25-cm) composed of Whatman 3MM paper were wet in pH 3.5 electrophoresis buffer (0.87 M glacial acetic acid, 0.5% (v/v) pyridine, 0.5 mM EDTA) and used to wet the TLC plate avoiding the area where the phosphoamino acids were located to prevent sample smearing. Once the plate was wet, the blotters were removed and the plate was rotated 90° counterclockwise, and electrophoresed 25 minutes at 1.3 kV in pH 3.5 electrophoresis buffer. After electrophoresis was complete, the plate was dried 20 minutes in an 80°C oven (Boyle *et al.*, 1991; Duclos *et al.*, 1991; Kamps, 1991).

II.3.11.5 Visualization of Phosphoamino Acids

In order to visualize the phosphoamino acid standards, the TLC plate was sprayed with a solution of 0.25% ninhydrin in acetone and then heated in an 80°C oven 10 minutes (Kamps, 1991). Once the standards were visible, they were traced in pencil on the backside of the plate since the purple color of the ninhydrin-stained phosphoamino acid standards faded with time. The positions of the stained phosphoamino acid standards were also traced onto a transparent sheet to serve as a template for comparison to the autoradiogram. Radioactive ink was used to make alignment marks on the TLC plate. The plate was wrapped in Saran wrap and exposed to a phosphorimager screen for at least 72 hours. Visualization was described previously.

II.4 Protein Purification

II.4.1 Column Chromatography

For column chromatography, Bio-Rad Econo-Pac polypropylene columns (1.5 x 12 cm) were packed with DEAE Sepharose FF. Preparation and equilibration of the column was carried out according to manufacturer's instructions. The packing and binding buffer used was 10 mM HEPES buffer at pH 7.5. Cell-free extract was labelled with [γ-^{32}P] ATP as described previously followed by acetone precipitation. After overnight precipitation, the samples were microcentrifuged 10 minutes to pellet the precipitated proteins and excess acetone was evaporated using a Speedvac. The protein pellets were dissolved in 500 μL of 10 mM HEPES and the protein solution was dialyzed with frequent changes of buffer for twelve hours at 4°C against 10 mM HEPES, or repeatedly acetone precipitated to remove unincorporated label. The protein was microcentrifuged before loading to ensure no particulates were present and loaded onto the ion-exchange column. A salt gradient was established by filling reservoirs with buffers of the same pH but different ionic strength. The mixing chamber contained the lower ionic strength buffer and the other chamber contained the higher ionic strength buffer. The maximum flow rate was 2 mL/min (1 drop/2 sec). The column was washed with 2 mL 10 mM HEPES before gradient elution with 250 mL 10 mM HEPES and 250 mL 10 mM HEPES + 1 M NaCl. Fractions representing 1% of the total gradient volume were collected using a Cygnet fraction collector. Fractions were collected and analyzed by liquid scintillation counting (LSC), SDS-PAGE and A_{280} (for cold fractions only). Conductivity was measured using a Cole-Parmer 01481-61 conductivity meter.

II.4.2 Electroelution of Proteins

The electroelution procedure of Hunkapillar *et al.* (1983) was used to purify the 32 kDa protein from SDS-polyacrylamide gels. The cell-free extracts were labelled as described previously except samples were heated at 60°C for 10 minutes prior to electrophoresis on a 10% SDS-PAG. Gels were stained with Coomassie Blue (0.5% (w/v) Coomassie Brilliant Blue R-250; 10% glacial acetic acid, 30% isopropyl alcohol) for 20 minutes at room temperature and then destained 3 hours to overnight in 5% glacial acetic acid, 16.5% methanol and autoradiographed. To avoid degradation of tryptophan and methionine side chains by free-radicals or oxidants trapped within the gel matrix, 0.1 mM sodium thioglycollate was added to the cathode buffer reservoir. The band of interest was located by alignment of the autorad and excised. Gel slices were placed in screw-capped tubes, counted in a Beckman LS 3801 liquid scintillation counter, then soaked in Milli-Q water on ice for two hours with frequent changes of water. Each wash was counted by liquid scintillation counting (LSC). Gel slices were placed in a petri dish and diced into ~1 mm cubes with a razor blade. The gel slices were rinsed briefly in soaking buffer (0.1% SDS, 0.05 M Tris acetate; pH 7.8) for 2 minutes. A #8 cork borer was used to cut a disk of dialysis membrane (Spectrafor MW cut off 6000-8000) which had previously been prepared by soaking 15 cm lengths of dialysis tubing in 1% $NaHCO_3$ at 60°C for one hour followed by a rinse in Milli-Q water. The tubing was then soaked in 0.1% SDS at 60°C for one hour and rinsed in Milli-Q water. The disk was placed on the small end of the Electrophoretic Elution system EE-04 (Tyler Research Instruments Edmonton, AB) and held in place with a rubber seal and screw cap. A one-inch square piece of tubing was placed over the large opening of the elution cell and held in place by rubber O-rings. Elution cells were filled with elution buffer and placed on a paper towel for 10 minutes before addition of gel pieces to inspect for leaks. Once gel pieces had been introduced into the elution cell, they were covered with elution buffer (2% SDS, 0.2 M Tris-acetate; pH 7.8). The slices were then overlaid with elution buffer to a level just above the drain ports in each electrode chamber and air bubbles were removed. The gel slices were allowed to soak at room temperature for 3 hours before the cell was inserted into the tank. A peristaltic pump was used to circulate buffer from the mixing chamber to the electrode chamber. Elution was carried out at 4°C at 100 V direct current for 16-20 hours. After this time, the elution buffer was replaced with dialysis buffer (0.02% SDS, 0.01 M NH_4HCO_3) and the eluate was dialyzed at 150 V direct current. Once complete, the eluted sample was collected and precipitated with two volumes of acetone overnight at –20°C. Samples were analyzed by SDS-PAGE. The eluted proteins were flash-frozen in a dry ice-ethanol bath and stored at –70°C

II.4.3 Sequential-Gel Band Purification Strategy for Peptide Microsequencing

Column chromatography proved to be problematic for the purification of the phosphorylated 32 kDa protein, therefore, a sequential-gel band purification strategy (Dr. K.L. Roy, personal communication) was employed. Two samples containing 200 µg total protein of [γ-^{32}P] ATP labelled cell-free extract of the 48 hour surface culture sample of *Streptomyces coelicolor* J1681 which showed significant phosphorylation of the 32 kDa protein, were electrophoresed on a 10% SDS-PAG. The assay buffer contained 10 mM $MnCl_2$ to enhance the phosphorylation state of this protein. Six similar but unlabelled samples were electrophoresed on the same gel. Sodium thioglycollate at 0.1 mM was added to the top reservoir buffer to scavenge any free radicals. The gel was stained briefly with Coomassie brilliant blue. The 32 kDa phosphorylated protein band of interest was located by comparison to pre-stained molecular weight markers and by autoradiography, was excised from the gel and colorized with a drop of bromophenol blue so that migration of the protein could be followed. A 15% SDS-polyacrylamide gel was poured around the excised gel strip. The gel was electrophoresed overnight at 100 V at 4°C. After electrophoresis, the gel was wrapped in Saran wrap and exposed to a phosphorimager screen for 1 hour. The gel was then stained for 10 minutes with Coomassie Blue (10% acetic acid, 30% isopropyl alcohol, 0.5% (w/v) Coomassie Brilliant Blue R-250) and was destained with 50% acetic acid, 16.5% methanol solution until the background was light blue. The destaining solution was decanted and a fine scalpel blade was used to excise the centre portion of the stained protein band ensuring no excess gel was excised. Gel slices of an equivalent area representing background were removed beneath the position of the 32 kDa band and were unstained indicating no visible protein was present at this level of detection. The excised bands were washed twice in 50% acetonitrile (HPLC grade) once for 5 minutes and once for 8 minutes in sterile Eppendorf tubes. Supernatants were removed as completely as possible and the tubes were flash frozen in dry ice-ethanol bath and stored at –70°C. The tubes were shipped on dry ice to the Harvard Microchemistry Facility for internal peptide sequencing.

II.5 Phage Manipulation

II.5.1 Single Plaque Soak-Out

The propagation of recombinant KC860 phage (Bruton *et al.*, 1991), a ϕC31-derived phage vector containing the promoterless *xylE* reporter gene, was accomplished by streaking a loopful of KC860 for isolation on Difco Nutrient Agar (DNA) supplemented with 0.5% glucose, 10 mM $MgSO_4$, 8 mM $Ca(NO_3)_2$ poured into 9 cm petri dishes which had been dried under laminar flow. These plates were overlaid with twenty microlitres of *S. lividans* 1326 spore stock (~10^7 spores) added to 12 mL of molten Soft Nutrient Agar (SNA). Plates were incubated at 30°C overnight (Hopwood *et al.*, 1985). Clear zones on the lawn of *S. lividans* 1326 indicated growth of phage KC860. Small, discrete plaques were chosen for single plaque soak out. A single plaque soak out was achieved by using a sterile Pasteur pipette to remove an agar plug from the middle of an isolated plaque. The agar plug was then ejected into 1.5 mL aliquot of sterile DNB. The phage were allowed to soak out for 2 hours, then the phage suspension was transferred to 1.5 mL sterile screw-capped tubes and stored at 4°C. DNB was used as diluent when dilution of a single plaque phage stock was necessary.

II.5.2 Preparation of *Streptomyces* Phage DNA

Phage KC860 (Bruton *et al.*, 1991) was cultivated overnight at 30°C in 2.5 mL SNA top layers containing *S. lividans* 1326 spores on 9 cm DNA plates inoculated with 0.1 mL DNB containing 2×10^4 PFU per plate. With this inoculum, almost confluent lysis was observed on the plate. A spreader was used to scrape top layers into aliquots of 25 mL DNB in sterile 100-mL beakers and the suspension was allowed to stand covered at room temperature for at least two hours. A 25 mL pipette was used to triturate the suspension twice during the incubation period. Once the incubation period was over, the phage-containing suspension was transferred to a 50 mL centrifuge tube and centrifuged at 16,000 x g for 10 minutes at 4 °C to remove the agar. In order to sediment the phage, the supernatant was transferred to a 30 mL polycarbonate centrifuge tube and centrifuged at 55,000 x g for 85 minutes at 4°C in Beckman model L7-55 ultracentrifuge. Once centrifugation was complete, the supernatant was discarded and the phage pellet was resuspended in 2 mL RNase solution (40 µg/mL DNase-free RNase heat treated in SM buffer which consisted of 20 mM Tris-HCl; pH 7.5, 1 mM $MgSO_4$, 100 mM NaCl, and 1.0 g/L gelatine) and incubated at 37°C on an orbital shaker for 30 minutes. The phage suspension was then transferred to 15 mL centrifuge tubes and 0.4 mL of SDS mix (1 volume 2 M Tris-HCl; pH 9.6, 2 volumes 0.5 M sodium EDTA; pH 7.4, and 1 volume 10% SDS) was added and incubated in a 70°C water bath for 30 minutes to disrupt the phage coat. After this incubation period, 0.5 mL potassium acetate was added and the tube was left on ice for 15 minutes to bring down the protein then centrifuged at 28,000 x g for 30 minutes at 2°C. The supernatant was then decanted into 50 mL polycarbonate centrifuge tubes followed by the addition of 7 mL TE buffer and 25 mL absolute ethanol. The tubes were placed at -20°C overnight then centrifuged at 7,800 x g for 10 minutes. The supernatant was discarded and the tubes were inverted on a Kimwipe for 10 minutes. The pellet was then resuspended in 1.0 mL TE buffer and precipitated with 1 volume 3 M sodium acetate and 2.5 volumes of absolute ethanol. The DNA was spooled onto a sealed, silanized Pasteur pipette and transferred to an eppendorf tube. The spooled DNA was dissolved in 270 µL of TE buffer and reprecipitated with 1 volume of 3 M sodium acetate and 2.5 volumes of absolute ethanol. The clot of DNA was then washed with

70% ethanol, dissolved in 100 µL sterile TE buffer and stored at 4°C (Hopwood *et al.*, 1985).

II.5.3 Transfection

Protoplasts of *S. lividans* 1326 were dispensed into round bottom screw cap tubes and centrifuged in a clinical centrifuge at 3000 rpm for 7 minutes. The supernatant was discarded and the protoplast pellets were resuspended in the drop of P Buffer remaining in the tube. A 5-10 µL aliquot of DNA (50-500 ng) was added to the protoplast suspension and 500 µL of 25% PEG solution was immediately added and triturated three times to mix the components. Aliquots of 0.1 mL of undiluted and 10-fold serial dilutions in P Buffer were plated on R2YE plates and overlayed with soft R2 agar (Hopwood *et al.*, 1985) containing *S. lividans* 1326 spores (1.5 mL overlay/5 cm plate). Plates were incubated at 30°C until plaques were evident in the *S. lividans* 1326 lawn. Plaques were observed where germinating spores were infected with phage.

With each transfection, a control using 50 ng of wild type ϕC31 DNA (Hopwood *et al.*, 1985) was also carried out (as described above) in order to calculate the transfection efficiency. KC860, which had been digested with BamHI and religated, was also used as a control to show that this vector alone forms plaques.

II.5.4 Lysogen Screening for *xylE* Activity

The overall scheme for identification of *bldA*-dependent promoters is shown in Figure III.1.1.1. Single plaque soak out (SPSO) preparations were prepared for all the plaques obtained from transfection of *S. lividans* 1326 protoplasts. These SPSOs were spotted onto master plates of Difco Nutrient Agar overlaid with *S. lividans* 1326 spores in SNA, allowed to grow and then were replica plated onto R2YE + TM plates which had been spread with a lawn of J1501 spores. Once these cultures had sporulated they were replica plated to Minimal Medium containing 1% glucose, 0.75% Tiger Milk (MMGTM) and thiostrepton at 50 μg/mL to select for lysogens. These plates were incubated at 30°C until colonies representing J1501 lysogens were observed. These plates were then sprayed with 0.5 M catechol. KC860 contains the promoterless *xylE* reporter gene (Zukowski *et al.*, 1983) which encodes the enzyme catechol 2,3 dioxygenase (C230) which catalyzes the production of 2-hydroxymuconic semialdehyde, a yellow colored product. Lysogens of interest turn yellow (Ylo^{+}) upon exposure to catechol while lysogens which do not contain inserts which activate *xylE* remain colorless (Ylo^{-}).

II.6 Protoplast Preparation

Protoplasts of *S. coelicolor* and *S. lividans* were prepared according the method described by Hopwood *et al.* (1985). Each 25 mL culture yielded ten 1 mL aliquots of protoplasts which were stored at -70°C. A single aliquot was used for each transformation.

II.7 Isolation of Nucleic Acids

II.7.1 Isolation of Chromosomal and Plasmid DNA

II.7.1.1 Isolation of *Streptomyces* Chromosomal DNA

Chromosomal DNA was isolated as described by Hopwood *et al.* (1985) using Procedure 1, which yielded DNA of high quality and was used routinely for cloning experiments. Procedure 3, a more rapid isolation method, was used for the verification of gene disruptants.

II.7.1.2 Isolation of *Streptomyces* Plasmid DNA

Small-scale plasmid preparations were performed as described in Sambrook *et al.* (1989) with the following modifications: lysozyme (2 mg/mL) was added to Solution I, and the mycelia in Solution I were incubated at 37°C for 30 minutes prior to the addition of Solution II.

II.7.1.3 Isolation of *E. coli* Plasmid DNA

The isolation of *E. coli* plasmids was performed as described in Sambrook *et al.* (1989).

II.7.2 Isolation of RNA

The modified procedure of Kirby *et al.* (1967) as described in Hopwood *et al.* (1985) was used for the isolation of *Streptomyces* RNA. Cultures were grown on the surface of cellophane disks on R2YE supplemented with Tiger's Milk. Mycelia were scraped off plates directly into universal bottles containing modified Kirby mixture plus glass beads, vortexed for two minutes in intervals of 30 seconds of vortexing, and 30 seconds on ice. The lysate was transferred with baked 5 mL glass pipettes to polystyrene tubes. Centrifugation was done at 8,500 rpm. The phenol:chloroform extraction was repeated at least three times until the interface was small. The initial isopropyl alcohol precipitation was at -70°C; the RNA was collected by centrifugation for 10 minutes. The pellet was washed with 1 mL of 95% ethanol and dissolved in 450 mL of diethyl pyrocarbonate (DEPC)-treated Milli-Q-water. The RNA was DNase treated twice with 70 units of RNase-free DNase at room temperature for 30 minutes, followed by two phenol:chloroform and two chloroform extractions. The RNA was precipitated in isopropyl alcohol on ice for 20 minutes. The purified RNA was dissolved in 100 µL of DEPC-treated Milli-Q-water and the absorbance at 260 nm of diluted samples were determined prior to storage in isopropyl alcohol at -70°C.

II.8 DNA Transformation

II.8.1 Transformation of *S. coelicolor*

Due to the restriction-modification system of *S. coelicolor*, DNA isolated from *E. coli* for transformation into *S. coelicolor* was passed through a *dam*$^-$, *dcm*$^-$ *E. coli* strain (ET12567). A 1 mL aliquot, containing approximately 4×10^9 protoplasts was transferred to a 15 mL round bottomed screw capped tissue culture tube and washed with 5 mL P buffer. A 10 µL aliquot of DNA in TE buffer was routinely used for transformations. Once DNA had been added to the protoplast suspension, 0.5 mL of 25% PEG 1000 in P buffer was added to assist the uptake of DNA, and 5 mL P buffer was added immediately. Following centrifugation, the protoplasts were resuspended in 0.5 mL P buffer and 0.1 mL aliquots were plated on R2YE plates supplemented with Tiger's Milk (Hopwood *et al.*, 1985). The plates were incubated at 30°C for 18 hours and overlaid with a 1 mL sterile suspension of thiostrepton in water to yield a final concentration of 50 µg of thiostrepton per mL of agar or overlaid with1 mL sterile solution of apralan in water to yield a final concentration of 50 µg of apramycin per mL of agar (apralan is approximately 50% apramycin). The plates were further incubated at 30°C until individual transformants were of suitable size to subculture. Transformants were subcultured on R2YE agar supplemented with Tiger's milk containing 50 µg/mL of thiostrepton or 50 µg/mL apramycin as appropriate.

II.8.2 Transformation of *E. coli*

E. coli DH5α competent cells were obtained from GIBCO-BRL and aliquoted (50 μL) into 1.5 mL eppendorf tubes, flash-frozen in a dry ice-ethanol bath and stored at –70°C.

E. coli ET12567 competent cells were prepared according to the procedure of (Tiong and Nash, personal communication). A 2.5 mL aliquot of an overnight culture of *E. coli* grown in 5 mL LB broth was added to 200 mL of prewarmed YT broth and incubated at 37°C on an orbital shaker until the OD_{600} reached 0.5 (~2 h, 25 min.). The culture was then separated into two 100 mL aliquots and centrifuged at 3,000 rpm for 10 minutes at 4°C. The pellets were resuspended in 40 mL of ice-cold solution A (30 mM potassium acetate, 50 mM $MnCl_2$, 100 mM KCl, 15% glycerol, 10 mM $CaCl_2$), pooled and incubated on ice for 1 hour. The cells were pelleted by centrifugation at 4°C for 10 minutes at 3,000 rpm and resuspended in 12 mL ice-cold solution B (10 mM MOPS; pH 7, 75 mM $CaCl_2$, 10 mM KCl, 15% glycerol). Aliquots of 200 μL were flash frozen in a dry ice-ethanol bath and stored at –70°C.

Transformation of *E. coli* was accomplished as follows: aliquots of competent cells were thawed on ice, 50 ng to 1 μg of plasmid DNA was added in 1-10 μL volumes and incubated for 30 minutes on ice. The cells were heat shocked for 45 seconds at 42°C for homemade competent cells and 20 seconds at 37°C for commercially prepared cells, and incubated on ice for 2 minutes. LB broth was added to a final volume of 1 mL and incubated at 37°C for 1 hour and plated onto LB plates containing either 100 μg/mL ampicillin or 50 μg/mL Apralan. To obtain blue-white selection where appropriate, LB agar containing antibiotic was supplemented with 40 μg/mL (Xgal) and 0.1 mM (IPTG). Transformants were incubated overnight at 37°C and subcultured on LB agar supplemented with antibiotic or in LB broth supplemented with antibiotic.

II.9 DNA Analysis

II.9.1 Restriction Digestion and Cloning of DNA

Restriction enzyme digestions of plasmid and chromosomal DNA were carried out according to Sambrook *et al.* (1989). Ligations of DNA with cohesive ends were carried out with a 2:1 or 3:1 molar excess of insert:vector in 10 μL volumes with 50-100 ng DNA/μL at 15°C overnight. Blunt-end ligations were carried out at room temperature (25°C) for 4 hours (King and Blakesley, 1986) with a 9:1 insert:vector ratio in 10 μL volumes. The 5X ligation buffer was composed of 250 mM Tris-HCl; pH 7.6, 50 mM $MgCl_2$, 25% PEG 8000, 5 mM DTT. ATP was added to the ligation reactions to a final concentration of 10 mM.

II.9.2 Agarose Gel Electrophoresis

DNA fragments in the size range of 0.7-8 kb were electrophoresed on 1% agarose gels using either a TBE buffer system (90 mM Tris, 89 mM Boric acid, 2.5 mM Na_2EDTA), or a TAE buffer system (40 mM Tris-acetate; pH 8.0, 1 mM EDTA). Molecular weight markers used were *Pst*I- or *Hin*dIII-digested λ DNA. Loading dye (0.25% bromophenol blue, 40% (w/v) sucrose) was added to samples so that migration could be followed. Gels were stained with ethidium bromide and exposed to an UV transilluminator to visualize the DNA bands.

II.9.3 Polyacrylamide Gel Electrophoresis

Fragments of DNA in the size range of 50-1000 bp were resolved by electrophoresis on 5% polyacrylamide gels (29:1 acrylamide: *N,N'*-methylene bisacrylamide) using a TBE buffer system. The molecular weight markers used were Molecular Weight Marker V (Boehringer Mannheim) or *Pst*I-digested λ DNA. Gels were stained in ethidium bromide and DNA bands were visualized on a UV transilluminator.

II.9.4 Colony Hybridization

Colony hybridization was used for the preliminary screening of large numbers of clones. For colony hybridizations of *E. coli* clones, the method described in Sambrook *et* *al.* (1989) was used. Grids were drawn on circular Hybond-N nylon membranes with a pen and autoclaved. The membranes were placed on LB agar plates supplemented with the appropriate antibiotic. A sterile toothpick was used to transfer colonies onto the membrane (50 per plate) as well as onto a duplicate master plate without a membrane. After overnight incubation at 37°C, the membranes were removed from the plate and placed colony-side up onto a piece of 3MM Whatman No. 1 filter paper saturated with denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 minutes. The membranes were then transferred to a piece of filter paper saturated with neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl; pH 7.2, 0.001 M Na_2EDTA) for 5 minutes, then placed in several volumes of 2X SSC (0.3 M NaCl, 0.03 M trisodium citrate) solution and the cellular debris was rubbed off. The filters were UV cross-linked at 150 mJoules in a Bio-Rad GS Gene Linker. Hybridization conditions and procedures were as described below.

Colony hybridizations for *Streptomyces* were accomplished on Whatman 541 filters (70 mm diameter) (Gergen *et al.*, 1979; Maas, 1983). The filters were divided into 9 equal squares, sterilized, and possible recombinant *Streptomyces* colonies were patched onto master plates as well as onto labelled filters which were placed on R2YE plates supplemented with Tiger's milk and the appropriate antibiotic. Plates were incubated at 30°C for 40 hours and the filters were peeled off the plates and soaked in TE buffer (10 mM Tris-HCl, 1 mM sodium EDTA) containing 4 mg/mL lysozyme for 10 minutes at 37°C (5 mL per filter). The filters were then immersed in boiling 0.5 M NaOH, 1% SDS for 5 minutes (20 mL/filter). The filters were neutralized in 0.5 M Tris-HCl, pH 7.5 for two washes of 5 minutes, immersed in 95% ethanol and air dried, followed by incubation in a vacuum oven at 80°C for 1 hour. Hybridization conditions were as described below.

II.9.5 Southern Hybridization

DNA was transferred from agarose gels to nylon membranes by an adaptation of the method of Southern (1975) and described by Hopwood *et al.* (1985). DNA fragments were separated by electrophoresis on a 1% agarose gel and stained as previously described. The gel, with gentle shaking, was then soaked twice for 10 minutes in 0.25 M HCl, twice for 15 minutes in denaturing solution (1.5 M NaCl, 0.5 M NaOH), rinsed three times in Milli-Q water and soaked in neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl; pH 7.2, 0.001 M Na_2EDTA) for 20 minutes. The gel was placed well-side down onto wicks consisting of two pieces of Whatman No. 1 filter paper saturated with 20X SSC (3 M NaCl, 0.3 M trisodium citrate) on a glass plate over a reservoir of 20X SSC. A Hybond-N nylon membrane was placed onto the gel followed by two additional pieces of Whatman filter paper, a stack of paper towels and a 0.5 kg weight. After overnight transfer the locations of the wells were marked onto the membrane with a solvent resistant pen and the membrane was UV cross-linked at 150 mJoules in a Bio-Rad GS Gene Linker.

Southern hybridization was carried out in 10-20 mL hybridization solution in glass hybridization tubes or in covered pyrex dishes. Prehybridization and hybridization solutions were composed of 3X SSC (0.45 M NaCl, 0.045 M $Na_3C_6H_5$), 4X Denhardt's solution (0.08% (w/v) Ficoll (MW 400,000), 0.08% (w/v) bovine serum albumin (Fraction V), 0.08% (w/v) polyvinyl pyrrolidone (MW 360,000), and 100 µg/mL denatured salmon sperm DNA. Prehybridization was allowed to proceed 6 hours to overnight while hybridizations were carried out overnight with two million cpm per 10 mL hybridization solution. Hybridization temperature was optimized for each probe. The dissociation temperature, T_d, of oligonucleotide probes was calculated by the formula: $T_d = 4(G+C) + 2(A+T)$ (Wallace *et al.*, 1979; Hopwood *et al.*, 1985). The melting temperature, T_m, of probes 50 bp or longer was calculated by the formula $T_m = 81.5° + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% \text{formamide}) - 500/L$, where *M* is the molarity of monovalent cations (0.45 M for 3X SSC), %GC is the percentage of guanine and cytosine nucleotides in the probe and *L* is the length of the duplex DNA segment in base pairs (Meinkoth and Wahl, 1984). Hybridizations were carried out 25° below T_m and 5° below T_d for duplexes that did not contain mismatches. For every 1% of mismatched bases for longer probes, the hybridization temperature was decreased 1° and 5° for every mismatched base for oligonucleotide probes. Formamide was added to both prehybridization and hybridization solutions to lower the T_m if the calculated hybridization temperature was greater than 65°C. Following hybridization, filters were washed at the hybridization temperature twice for 30 minutes in 2X SSC, 0.1% SDS and twice for 30 minutes in 0.2X SSC, 0.1% SDS. Filters were then wrapped in Saran wrap and exposed to Kodak X-OMAT AR film with an intensifying screen at –70°C or alternatively exposed to a phosphorimager screen at room temperature as previously described. Membranes were stripped by immersion in boiling 0.1% SDS and cooled to room temperature. This was repeated until no signal was detectable upon exposure to either film or the phosphorimager screen.

II.9.6 ^{32}P-Labelling of Probes

II.9.6.1 Kinase Labelling

Oligonucleotide probes were 5' end-labelled with [γ-^{32}P] ATP by the procedure described by Chaconas and van de Sande (1980). Twenty picomoles of oligonucleotide primer was incubated with 1 µL of 10X kinase buffer (0.5 M Tris-HCl; pH 8.0, 0.1 M $MgCl_2$, 50 mM DTT, 1 mM spermidine), 5 µL (50 µCi) of [γ-^{32}P] ATP, and 1 µL of 1:10 diluted polynucleotide kinase (dilution buffer consisted of 50 mM Tris-HCl; pH 8.2, 1 mM DTT, 0.1 mM EDTA, 50% glycerol) in a total volume of 10 µL. The reaction was incubated at 37°C for 30 minutes followed by the addition of another aliquot of diluted kinase and incubated for a further 30 minutes. Either Nuc-Trap® or Micro Bio-spin® probe purification columns were used to purify probes. One-microlitre aliquots were counted in a Beckman LS 3801 scintillation counter.

II.9.6.2 Random Primer Labelling

Probes generated from double-stranded DNA fragments were internally labelled with [α-^{32}P] dCTP by the random primer labelling method described by Feingberg and Vogelstein (1983). In a screw-capped tube, 50-500 ng of template DNA in a volume of 9 µL was denatured at 90°C and chilled on ice. This was followed by the addition of 2 µL hexanucleotide mix, 3 µL 1.5 mM dNTP mix (dATP, dGTP, dTTP), 5 µL (50 µCi) of [α-^{32}P] dCTP, 2 units of Klenow and incubated for 4 hours at 37°C or overnight at room temperature. Labelled probes were purified by the use of Nuc-Trap®, or Micro Bio-spin® probe purification columns. One-microlitre aliquots were counted in a Beckman LS 3801 scintillation counter to assess labelling. Random labelled probes were denatured for 5 minutes at 95°C immediately prior to use.

II.9.7 DNA Sequence Analysis

II.9.7.1 ^{33}P- Sequencing

DNA sequence analysis was performed using the chain termination method of Sanger *et al.* (1977) as modified by Tabor and Richardson (1987) for use with ThermoSequenase™ radiolabelled terminator cycle sequencing kit using [α-^{33}P] ddNTPs. Template DNA was prepared as described in section II.8.1.3. ^{33}P-labelled fragments were separated on 6% denaturing polyacrylamide gels using a TBE buffering system. Electrophoresis was carried out at 35 Watts for 2-5 hours. After electrophoresis, gels were fixed in 10% methanol, 10% acetic acid for 10 minutes, lifted onto 3MM Whatman No. 1 filter paper and dried for 2 hours under vacuum at 80°C in a Bio-Rad Model 583 Gel Drier. Radioactive bands were visualized by exposure of the sequencing gels to Kodak X-OMAT AR film at room temperature for 1-3 days, or by exposure of the gel to a phosphorimager screen.

II.9.7.2 Automated Sequence Analysis

Double-stranded plasmid DNA of most clones generated in this study were sequenced by the Department of Biological Sciences Sequencing Service (University of Alberta).

II.10 Purification of DNA Fragments

II.10.1 Recovery of DNA from Agarose Gels

Agarose gels were routinely used for the purification of DNA fragments 300 to 5000 bp in size by the trough method of Zhen and Swank (1993). DNA fragments were separated in an agarose gel (0.8%-1.2% agarose) in TAE buffer containing 0.1 μg/mL ethidium bromide (EtBr). The band of interest was visualized by illumination with long-wave ultraviolet light. A rectangular trough was excised from directly in front of the band. The resulting trough was filled with 15% PEG/TAE/EtBr buffer. Electrophoresis was allowed to continue with the level of electrophoresis buffer adjusted so that it did not cover the top of the gel. A long-wave UV lamp was used to follow mobility of the fragment of interest. When the DNA band of interest had moved into the centre of the trough, electrophoresis was stopped and the DNA-containing PEG/TAE solution was retrieved from the trough and transferred to an Eppendorf tube. To remove the PEG, the solution was subjected to phenol and chloroform extractions followed by ethanol precipitation. The DNA pellet was rinsed with 1 mL 70% ethanol prior to dissolution in TE buffer (Zhen and Swank, 1993).

II.10.2 Recovery of DNA from Polyacrylamide Gels

Fragments of DNA up to 1 kb were isolated from polyacrylamide gels using the "crush and soak" method described in Sambrook *et al.* (1989). The polyacrylamide gel slice was placed in an eppendorf tube with 300 µL of elution buffer (0.5 M ammonium acetate, 1 mM EDTA; pH 8.0, 10 mM magnesium acetate, 0.1% SDS); the polyacrylamide gel slice was crushed with an eppendorf tube grinder and the DNA was eluted overnight at 37°C in a tube roller. To recover the supernatant, the Eppendorf tube was microcentrifuged at 15,000 rpm for 5 minutes to pellet the acrylamide, and the supernatant was transferred into a fresh Eppendorf tube with a gel-loading tip. The acrylamide was then extracted with an additional aliquot of elution buffer and the supernatant was removed after vortexing and microcentrifugation. The pooled supernatants were centrifuged a second time to remove any remaining acrylamide, and the DNA was ethanol precipitated.

II.11 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was used to amplify DNA fragments for cloning, and to generate probes for hybridization, sequencing, and transcript mapping.

The Actinomycete protocol for PCR (described by Eung-Soo Kim: http://molbio.cbs.umn.edu/asirc/protocol/pcrhighgc.html) achieved the best results especially for problematic amplifications. Reactions were carried out in 100 µL volumes in 0.5 mL eppendorf tubes using a Techne PHC-2 thermocycler. Reactions contained 150 pmol of each primer; 0.2 mM dNTPs; 5% DMSO; 1.5 mM $MgCl_2$; 10 µL 10X Buffer 3; 10 ng of plasmid template or 1 µg of denatured chromosomal DNA template and 2.5 units of Expand™ polymerase. Reactions were overlaid with 2 drops sterile mineral oil and denatured for 5 minutes at 95°C, followed by 30 cycles of 30 seconds at 95°C, 1 minute at 37°C, and 2 minutes at 72°C. Prior to use as template, chromosomal DNA was first denatured as described by Agarwal and Perl (1993) in 0.4 M EDTA; pH 8.0, 0.4 N NaOH for 10 minutes at room temperature.

For non-problematic PCR, 100 µL reactions contained 20 pmol of each primer, 0.2 mM dNTPs, 0-5% DMSO, 10 µL 10X Buffer 1, 1 µg denatured chromosomal DNA template and 2.5 units of Expand polymerase. Reactions were overlaid with 2 drops of sterile mineral oil and denatured for 5 minutes at 95°C, followed by 30 cycles of 30 seconds at 95°C, 30 seconds at 45-68°C, 1 minute at 72°C. The annealing temperature was adjusted to 5°C below the T_d of the primer with the lower T_d.

II.12 RNA Analysis

II.12.1 RNA Agarose Gel Electrophoresis

RNA transcripts were subjected to electrophoresis on 1.25% agarose gels using a 10 mM sodium phosphate buffer system (Williams and Mason, 1985). Forty microgram samples of RNA in isopropyl alcohol were precipitated by microcentrifugation, washed with 80% DEPC-treated ethanol and dissolved in 2.5 µL of DEPC-treated water. The samples were denatured with 2 µL glyoxal, 6 µL DMSO and 1.5 µL 80 mM sodium phosphate buffer, pH 6.5 for 60 minutes at 50°C, and chilled on ice. The molecular weight markers used were *Pst*I- and *Hin*dIII-digested λ DNA, which were also denatured with deionized glyoxal, and DMSO as described above. Three microlitres of loading dye (50% glycerol, 10 mM $NaPO_4$; pH 7, 0.4% bromophenol blue) were added to both the RNA samples and molecular weight markers prior to loading. Denatured samples were separated by electrophoresis at 4 V/cm for 4 hours with recirculation of buffer.

II.12.2 Transfer of RNA to Nylon Membranes

Northern transfer of RNA resolved on agarose gels was accomplished by capillary blotting overnight in 20X SSC to Hybond-N nylon membranes. After transfer, the wells were marked on the membrane with solvent resistant pen, UV cross-linked at 150 mJoules in a Bio-Rad GS Gene Linker and baked in an 80°C vacuum oven for 2 hours. The molecular weight marker lanes were cut off the membrane and either stained with

0.2% methylene blue, 0.2 M sodium acetate; pH 4.7, (Gerard and Miller, 1986) and destained with Milli-Q water or probed with the appropriate radiolabelled marker.

II.12.3 Northern Analysis

Hybridization for northern analysis was performed in either glass hybridization tubes or covered Pyrex dishes in ten to twenty millilitres of hybridization solution. Prehybridization reactions were carried out six hours to overnight while hybridization was allowed to proceed overnight with 2 million cpm probe per 10 mL of hybridization solution. Hybridization conditions were optimized for each probe as described previously. For hybridizations with longer probes, formamide buffer (50% formamide, 6X SSC, 1X Denhardt's, 0.1% SDS) was used to lower the T_m. Filters were washed at the hybridization temperature twice for 30 minutes in 2X SSC, 0.1%SDS and twice for 30 minutes in 0.2X SSC, 0.1%SDS. The membranes were wrapped in Saran wrap and exposed to Kodak X-OMAT AR film with intensifying screen at –70°C, or exposed to a phosphorimager screen at room temperature. Blots were stripped at 65°C in 0.5 mM Tris-HCl; pH 8.0, 2.0 mM EDTA, 0.1X Denhardt's for 2 hours (Amersham Manual) until no signal could be detected by overnight exposure to a phosphorimager screen. As a control for RNA loading levels, BKL54 (5'-CCGCCTTCGCCACCGGT-3'), a probe for 16S rRNA corresponding to a conserved region of *Streptomyces* 16S rRNA sequences (Baylis and Bibb, 1987), was hybridized to the same blot.

II.12.4 *S1* nuclease Protection Assays

S1 nuclease protection assays were performed to identify the transcription start point of the succinyl Co-A synthetase (SCS) operon. The probes used for S1 mapping were generated by PCR using primers designed to span the putative transcription start site and had a non-homologous 5' end which eliminated the need to separate the two strands of the PCR generated probe. The non-homologous extension was removed by *S1* nuclease treatment and was not expected to result in the appearance of labelled protected fragments. The probe for *S1* nuclease mapping of the *sucD* transcription start site was generated by PCR amplification of a 241 bp fragment using denatured J1501 chromosomal DNA as template. The primers were an 18-mer synthetic oligonucleotide JLS54 (5'-GTGTGCTTCATGCCCTCG-3') internal to the *sucD* coding sequence and a 28-mer synthetic oligonucleotide JLS56 (5'-GCGCAAGCTTCTCACCAAACCGCTCGTC-3') corresponding to a region 185 bases upstream of the translation start of *sucD*. The upstream primer, JLS56, contains a 10 nucleotide non-homologous extension (underlined) at the 5' end. The probe for *S1* nuclease mapping of the *sucC* gene was generated by PCR amplification of a 301 bp fragment (Probe A) using denatured J1501 chromosomal DNA as template. The primers were an 18-mer synthetic oligonucleotide JLS53 (5'-TCCCGTGTTCCTTGAAGA-3') corresponding to an internal region of the *sucC* coding sequence and a 28-mer synthetic oligonucleotide JLS55 (5'-GCGCAAGCTTACGCAGCCGTCGTAGATG-3') corresponding to the region 272 bases upstream of the translation start of the *sucC* coding sequence. The upstream primer contains a 10 nucleotide non-homologous extension (underlined) at its 5' end. RNA samples (5-30 µg) were hybridized to 50,000-200,000 cpm of [γ-^{32}P] ATP end-labelled probe in formamide hybridization buffer (3.2 mM PIPES buffer; pH 6.4, 0.4 M NaCl, 1 mM EDTA, 80% (v/v) deionized formamide). The samples were incubated at 80°C for 30 minutes, then slowly cooled to the annealing temperature (5°C above the Tm of the expected DNA/RNA hybrid). The samples were chilled on ice and treated with 150 units of S1 nuclease in digestion buffer (0.28 M NaCl, 30 mM sodium acetate; pH 4.4, 4.5 mM zinc acetate, and 20 μg denatured calf thymus DNA) for 45 minutes at 37°C. Termination solution (2.5 M ammonium acetate, 0.05 M EDTA) was added to stop the reactions (Hopwood *et al.*, 1985). The reactions were precipitated with 1/10 volume of 3M sodium acetate, 2 μL glycogen and 2 volumes of 95% ethanol for 30 minutes on ice. The pellets were redissolved in 5 μL loading dye (98% deionized formamide, 10 mM EDTA; pH 8.0, 0.025% xylene cyanol, 0.025% bromophenol blue) and denatured for 5 minutes at 95°C prior to loading on a 6% denaturing polyacrylamide gel. Sequencing ladders for identification of each transcript start point were generated using the PCR primer that was predicted to hybridize within the transcript.

II.12.5 Primer Extension

Primer extension reactions were performed to identify the transcription start site of the genes encoding the subunits for SCS. Primers were designed to hybridize approximately 100 bases from the proposed 5' end of the transcript. Fifty picomoles of primer were end-labelled with [γ-^{32}P] ATP as previously described. Five picomoles of primer was annealed to 5-20 μg of RNA in 2 μL 10X SB buffer (600 mM NH_4Cl, 100 mM Tris-acetate; pH 7.4, 60 mM β-mercaptoethanol) (Hartz *et al.*, 1988) in a total reaction volume of 20 μL. The reactions were then incubated at 90°C for 5 minutes, transferred to a 75°C water bath, and allowed to slow cool to 55°C. After incubation, reaction tubes were pulsed and chilled on ice. Five microlitres of the annealing reaction were added to reverse transcription (RT) assay tubes containing 1 μL of 10X SB buffer, 1.5 μL 100 mM magnesium acetate, 6 μL 5 mM dNTP mix, 0.5 μL DEPC-water, 0.5 μL RNA-guard, and 12.5 units AMV-RT. Reactions were incubated at 45°C for 30 minutes after which time 2.0 μL formamide loading dye was added. Reactions were denatured 10 minutes at 80°C and 4.5 μL of the sample was loaded onto a 6% denaturing polyacrylamide gel (Kelemen *et al.*, 1991; Stern *et al.*, 1988) alongside sequencing ladders generated using the same primer used for the annealing reaction.

II.12.6 Two-Step Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

As a means of confirming results obtained by Northern analysis, two-step RT-PCR was performed using the *C. therm* polymerase two-step RT-PCR kit (Boehringer Mannheim). As a control for RNA loading, RT-PCR of constitutively expressed *hrdB* was carried out using DBG6 (5'-GCCAAGAAGAACCACCGCCAAG-3') and DBG7 (5'-GACCTTGCCGATCTGCTTGAG-3'). PCR amplification with DBG6 and DBG7 results in a product of 215 bp. The RT reactions were carried out in thin-walled 0.2 mL PCR tubes. Each 20 μL reaction contained 4 μL of 5X RT buffer, 1 μL DTT, 0.64 μL 25 mM dNTPs, 0.6 µL DMSO, 20 pmol DBG7, 0.5 µL RNA guard, 9.76 µL DEPC-water, 3 µg RNA and 4 units of *C. therm* polymerase. Reactions were incubated for 30 minutes at 69°C with a hot bonnet, eliminating the need for mineral oil. Master mix I contained 4 µL DMSO, 0.4 µL 25 mM dNTPs, 15 pmol [γ-^{32}P] ATP end-labelled DBG7, 15 pmol DBG6, 12.1 µL DEPC-water, and 5 µL template cDNA. Master mix II contained 5 µL 10X Buffer 3, 10 µL betain, 9.25 µL Milli-Q water, 2.5 units Expand DNA polymerase mix. Twenty-five microlitres of master mix I and master mix II were added together in a fresh 0.2 mL thin-walled PCR tube. The PCR conditions consisted of denaturation for 2 minutes at 95°C followed by 30 cycles of 30 seconds at 95°C, 30 seconds at 58°C, and 1 minute at 68°C. The final PCR cycle was a 7-minute incubation at 70°C. Loading dye (5 µL) was added to the reactions and 15 µL aliquots were subjected to electrophoresis on a 5% polyacrylamide gel with a TBE buffer system. Two-step RT-PCR was also carried out for *sucD* using JLS46 (5'-GGCTAAGCTTGTCACGGTCGTCTTCGTG-3') and JLS47 (5'-CCACTCTAGACATCGTCCGGCCCCTCG-3'). The procedure was identical to that used for RT-PCR of *hrdB* with the following exceptions: JLS47 was used in the RT reaction, [γ-^{32}P] ATP end-labelled JLS47 was used in master mix I.

III RESULTS

The manipulation of antibiotic production relies on understanding the nature of the regulatory components involved in the induction of genes responsible for antibiotic biosynthesis. Mutants, such as the *bldA* mutants, which are defective in their ability to sporulate and in their ability to produce antibiotics, must have some defect in the synthesis of key regulators involved in both processes. In this study, efforts were made to identify *bldA*-targets. These targets are TTA-containing genes that require the *bldA*-encoded tRNA for their translation. Since the TTA codon for leucine is confined to a small number of *Streptomyces* genes, which are primarily engaged in the regulation of morphogenesis and secondary metabolism, these targets would very likely have implications in the regulation of developmental events. The search for these targets was not trivial since they were expected to be few in number, and since there was no simple way to screen for a TTA-containing gene.

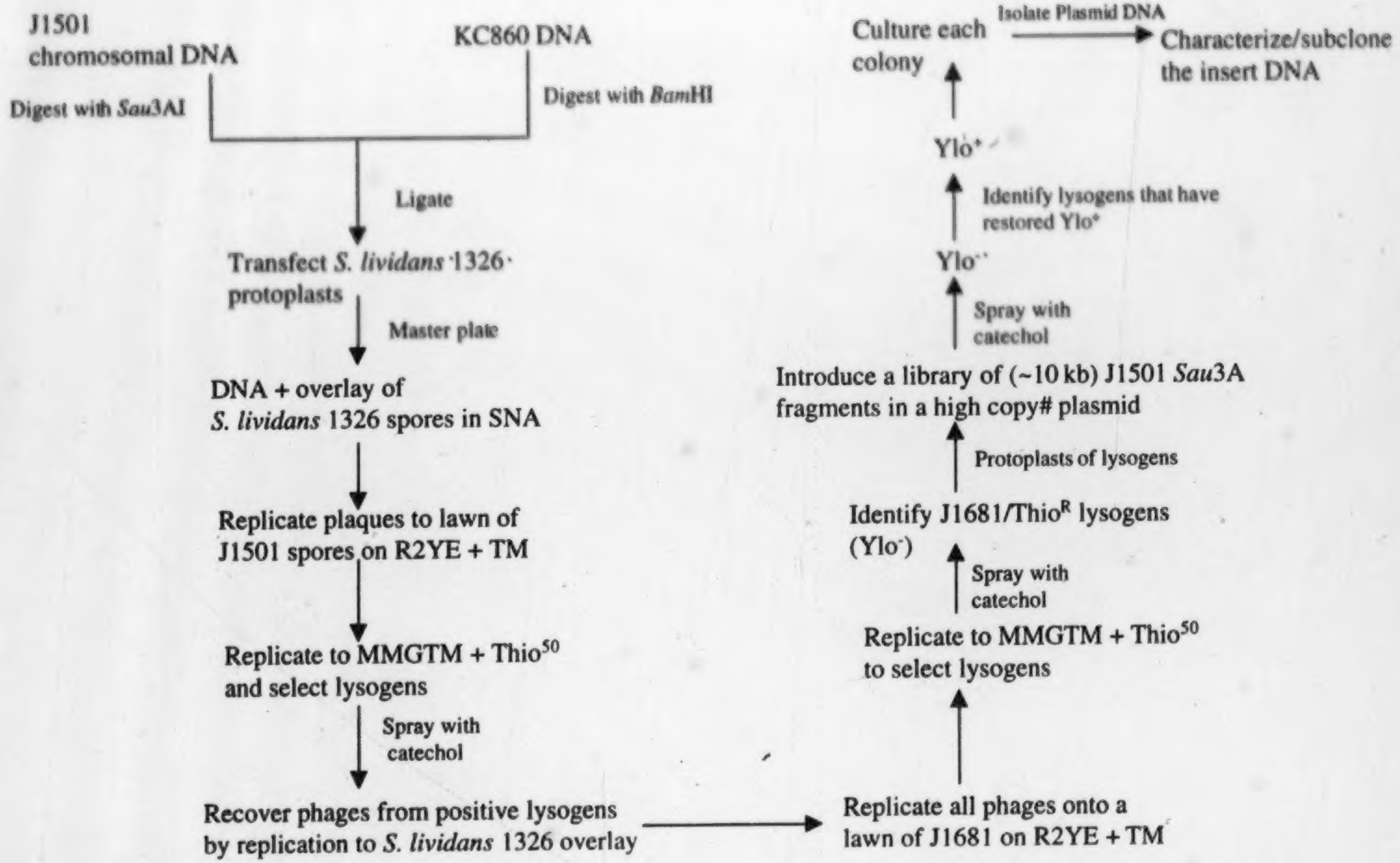
III.1 Genetic Approach To Cloning TTA-Containing Regulatory Genes

Previous research had determined that the expression of some structural genes for the antibiotic actinorhodin was reduced in *bldA* mutants (Guthrie and Chater, 1990), (Bruton *et al.*, 1991) and that actinorhodin production in *bldA* mutants could be restored by introduction of the cloned TTA-containing positive regulatory gene on a high copy number vector without restoration of aerial mycelium formation (Passantino *et al.*, 1991). This seemed to suggest it might be possible to identify TTA-containing regulatory genes by screening for clones that restored expression of reporter genes from transcriptional fusions in a *bldA* mutant by using a library of promoter fusions to a reporter gene. Promoters which show *bldA*-dependent expression are likely to require a TTA-containing gene as an activator or as a repressor of transcription. If TTA-containing regulators are cloned at high copy number, enough mistranslation might occur to allow the expression of a few molecules of the activator which may then be assayable using reporter fusions. To accomplish this, an attempt was made to identify promoters showing altered regulation in a *bldA*$^-$ strain.

III.1.1 Attempted Identification of Promoters Showing *bldA*-Dependent Regulation

In an attempt to identify promoters showing *bldA*-dependent regulation, chromosomal DNA from J1501 (*bldA*$^+$) was partially digested with *Sau*3AI to generate random fragments (Figure III.1.1.1). These fragments were then ligated into KC860 (Bruton *et al.*, 1991), the *Streptomyces* ϕC31-derived phage vector containing the promoterless *xylE* reporter gene, which had been digested with *Bam*H1. KC860 does not possess an *attP* site and will therefore integrate into the chromosome by recombination with sequences homologous to the insert DNA. The *xylE* gene of *Pseudomonas putida* (Zukowski *et al.*, 1983) has been shown to be a useful reporter gene in *S. lividans* (Ingram *et al.*, 1989) as well as *S. coelicolor* A3(2) (Guthrie and Chater, 1990). The *xylE* gene was chosen as a reporter because it does not contain TTA codons and because its expression is not dependent on the age of the culture as are the antibiotic resistance reporter genes (Ward *et al.*, 1986).

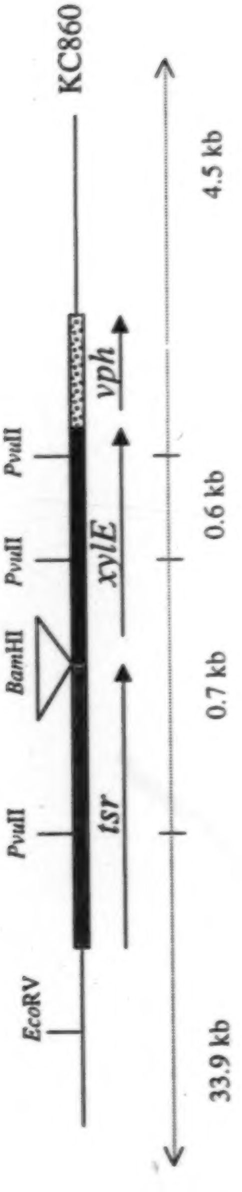
Figure III.1.1.1. Flow diagram of proposed experimental steps for the identification of promoters showing *bldA*-dependent regulation. Abbreviations: DNA, Difco Nutrient Agar; TM, Tiger's Milk; MMGTM, Minimal medium supplemented with glucose and Tiger's milk; SNA, Soft Nutrient Agar; Thio^{50}, thiostrepton at a final concentration of 50 μg/mL; Ylo, yellow.


J1501
chromosomal DNA
KC860 DNA
Digest with *Sau*3AI
Digest with *Bam*HI
Ligate
Transfect *S. lividans* 1326 protoplasts
Master plate
DNA + overlay of *S. lividans* 1326 spores in SNA
Replicate plaques to lawn of J1501 spores on R2YE + TM
Replicate to MMGTM + $Thio^{50}$ and select lysogens
Spray with catechol
Recover phages from positive lysogens by replication to *S. lividans* 1326 overlay
Replicate all phages onto a lawn of J1681 on R2YE + TM
Replicate to MMGTM + $Thio^{50}$ to select lysogens
Spray with catechol
Identify J1681/$Thio^R$ lysogens (Ylo^-)
Protoplasts of lysogens
Introduce a library of (~10 kb) J1501 *Sau*3A fragments in a high copy# plasmid
Spray with catechol
Ylo^-
Identify lysogens that have restored Ylo^+
Ylo^+
Culture each colony
Isolate Plasmid DNA
Characterize/subclone the insert DNA

The optimal DNA concentrations for ligation were found to be 1 µg KC860 and 25 ng of J1501 insert DNA. These ligations were used to transfect *S. lividans* 1326 protoplasts. Single plaque soak outs of the 7,000 plaques obtained were prepared and spotted onto plates containing a lawn of J1501 to generate lysogens. Figure III.1.1.2 shows a diagrammatic representation of KC860. Insertion of a fragment into the *Bam*HI site would increase the size of the 0.7 kb band generated by *Pvu*II digestion. Twelve random plaques were screened by digestion with *Pvu*II and electrophoresis on a 1% TAE agarose gel. Eight out of the twelve plaques were found to contain inserts.

Lysogens obtained from the successful transfection experiments were further screened by replica plating onto selective medium (MMGTM supplemented with 50 µg/mL thiostrepton) and sprayed with 0.5 M catechol in an effort to identify the insert-directed activation of *xylE*. In the presence of catechol, catechol 2,3 dioxygenase (C23O) catalyzes the production of 2-hydroxymuconic semialdehyde, a yellow colored product (Zukowski *et al.*, 1983). Lysogens which contain inserts internal to a transcription unit, inserts that will cause integration into the chromosome such that a chromosomal promoter will drive the transcription of *xylE*, or inserts that contain a promoter, are expected to turn yellow upon exposure to catechol (Ylo$^+$). Lysogens which do not possess inserts or which have inserts lacking promoter sequences, or contain inserts with promoter sequences in the wrong orientation, will not activate *xylE* and will not undergo a color change (Ylo$^-$).

Figure III.1.1.2. Diagrammatic representation of the right arm of the ϕC31::*xylE* vector, KC860. The left arm of this vector is identical to ϕC31. Solid arrows represent the direction of transcription. Abbreviations: *tsr*, gene for thiostrepton resistance; *xylE*, gene for the product catechol 2,3 dioxygenase; *vph*, gene for viomycin resistance.


EcoRV
PvuII
BamHI
PvuII
PvuII
KC860
tsr
xylE
vph
33.9 kb
0.7 kb
0.6 kb
4.5 kb

Streptomyces lividans TK24/pIJ4083/*ermE** was used as a positive control. The plasmid, pIJ4083/*ermE**, has the *xylE* gene under control of *ermE**, the up-promoter mutant form of the promoter for the erythromycin resistance-encoding gene (Petrich *et al.*, 1992). This strain turned yellow within two minutes when sprayed with 0.5 M catechol due to the production of 2-hydroxymuconic semialdehyde. S. *lividans* TK24/pIJ4083 was used as a negative control. The pIJ4083 plasmid (Clayton and Bibb, 1990) contains the promoterless *xylE* gene; it did not produce 2-hydroxymuconic acid and therefore was not yellow after cultures were sprayed with catechol. Lysogens of J1501::KC900 were also sprayed with catechol as a positive control. KC900, a derivative of KC860, contains a 0.9 kb fragment internal to the *actI* transcription unit inserted in the same orientation as *xylE*. Integration of KC900 into the chromosomal *actI* region disrupts the transcription unit at its 5' end (Bruton *et al.*, 1991; Malpartida and Hopwood, 1986). The lysogens, which contain a disrupted *actI* region of the chromosome do not produce actinorhodin but do exhibit a Ylo$^+$ phenotype when sprayed with catechol (Bruton *et al.*, 1991) because *xylE* is driven by the upstream *act*I promoter. As a final control, J1501 was transfected with KC860, which is *att*$^-$ and therefore cannot integrate into the chromosome, to show that it was not possible to generate thiostrepton resistant lysogens.

Phages released from yellow lysogens were to have been replicated onto a lawn of spores from a *bldA*$^-$ strain and the level of *xylE* expression was to have been compared to that of a *bldA*$^+$ strain (Figure III.1.1.1). However, screening by replica plating onto a selective medium (MMGTM supplemented with 50 µg/mL thiostrepton) and spraying with catechol did not prove to be effective. The lysogens, by the time they had sporulated, produced a brown pigment that masked any yellow color that may have been produced by insert-activation of *xylE*. When younger, non-sporulating cultures were sprayed with catechol, a color change was not observed. The positive control strains required a significant amount of growth for visualization of the yellow color so it was possible that younger cultures did not possess adequate biomass. By the time biomass had accumulated to levels equivalent with those of the positive control strains, the brown pigment was already being produced. To determine if the pigment was a result of some component in the medium, different media were tested. A compromise between adequate growth and lack of pigment production could not be found. No attempt was made to identify or characterize the brown pigment. Although extracts could have been made of each of the lysogens for use in a colorimetric assay, it was decided that this method would be too labour intensive to screen such a large number of lysogens and so alternative methods were pursued.

A method had been previously described whereby Ylo$^+$ and Ylo$^-$ plaques were identified by transferring plaques to R2 agar carrying a double top layer of SNA, one containing 2 mM catechol and one containing 10^7 *S. lividans* spores per mL and 2 mM catechol (Bruton *et al.*, 1991). Base plates of R2 and R2YE supplemented with Tiger's milk (Hopwood *et al.*, 1985) were prepared containing either 2 mM catechol or 5 mM catechol. The double top layer of SNA was prepared as described above. After transferring the plaques onto the plates, plates were incubated in the dark overnight at 30°C. It was found that *S. lividans* grew very poorly on this medium (likely inhibited by the presence of catechol) and that the medium turned from a pink to dark brown color upon incubation at 30°C even when plates were wrapped in foil making this an inappropriate method for screening plaques.

In light of these results, a reverse-genetic approach was taken to try to identify potential *bldA* targets.

III.2 One-Dimensional SDS-Page for Comparison of Protein Profiles

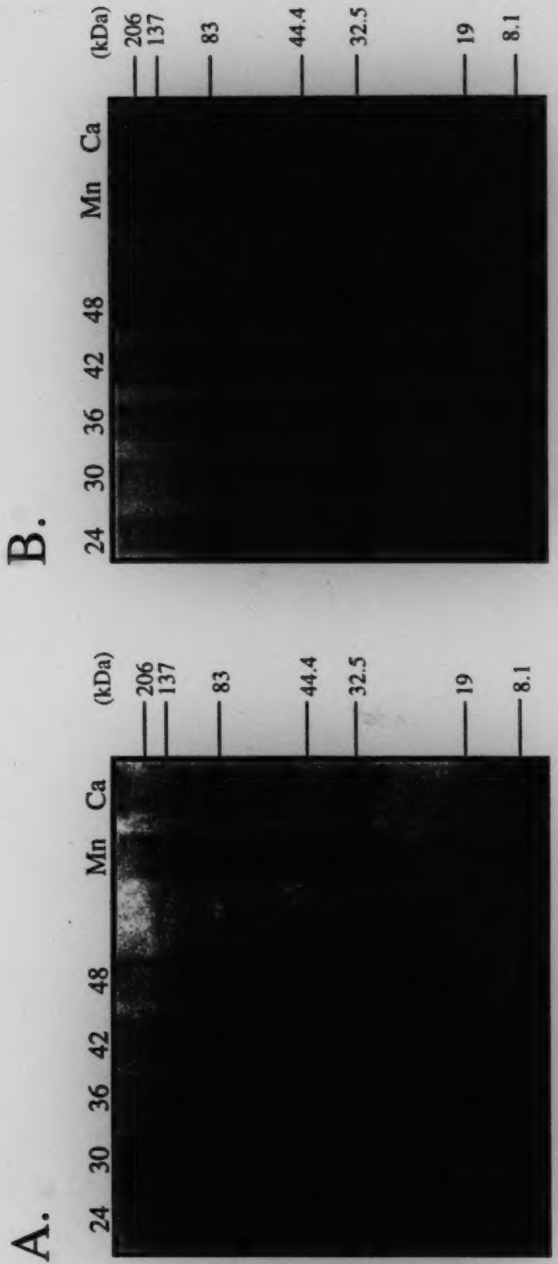
Efforts aimed at identifying *bldA*-targets utilizing a genetic approach were less than satisfactory, therefore, the search for targets became focused on the comparison of protein profiles between *S. coelicolor* J1501 (*bldA*$^+$) and *S. coelicolor* J1681 (*bldA*$^-$). Liquid and surface culture time courses were performed and cell-free extracts (CFEs) were prepared as previously described. The protein profiles generated from cell-free extracts of the *bldA*$^+$ and the *bldA*$^-$ strain were compared by both one- and two-dimensional SDS-polyacrylamide gel electrophoresis in order to identify proteins that were *bldA*-dependent. Due to the complexity of unlabelled CFE protein profiles, with greater than 2000 protein spots per two-dimensional gel, the search for *bldA* targets (TTA-containing genes) was narrowed by the use of *in vitro* protein phosphorylation assays to identify proteins phosphorylated by ATP during chemical and morphological differentiation of *S. coelicolor*. Phosphorylation was chosen because phosphorylation cascades have been implicated in the regulation of the onset of sporulation in *Bacillus subtilis* (Burbulys *et al.*, 1991).

Since *Streptomyces coelicolor* does not undergo morphological differentiation (sporulation) in liquid culture, it was of interest to ascertain any differences in temporal and phosphorylation intensity of proteins extracted from liquid cultures compared to those extracted from surface cultures between the *bldA*$^+$ and the *bldA*$^-$ strain.

III.2.1 Liquid Culture

Liquid culture cell-free extracts obtained from different stages of growth of the isogenic strains J1501 (*bldA*$^+$) and J1681 (*bldA*$^-$) were prepared. To narrow the number of candidates for potential *bldA* targets from the complex pool of possibilities in whole cell-free extracts, extracts were labelled *in vitro* with [γ-^{32}P] ATP as described by Wang and Koshland (1981) and electrophoresed on 10% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, and phosphorylation profiles were visualised by exposure to film at –70°C with an intensifying screen (Plate III.2.1.1). Since it is known that manganese and calcium ions play a role in the phosphorylation of proteins (Hong *et al.*, 1993; Natsume *et al.*, 1989; Weinberg, 1977a), it was therefore of interest to determine if these ions had an effect on the phosphorylation profiles of J1501 and J1681. To address this, the 48-hour cell-free extracts were subjected to phosphorylation in reactions supplemented with 10 mM $MnCl_2$ and 30 mM $CaCl_2$ (Plate III.2.1.1). These concentrations were used because they were found to be optimal for *S. griseus* (Hong *et al.*, 1993). There are only a few phosphorylated proteins observed when whole CFEs were labelled by this procedure (Plate III.2.1.1) thus significantly reducing the number of potential *bldA* targets to a more manageable number.

Plate III.2.1.1. Protein phosphorylation profiles for *Streptomyces coelicolor* (A) J1501 (*bldA*$^+$) and (B) J1681 (*bldA*$^-$) grown in liquid cultures. Whole cell-free extracts were prepared from cultures harvested at 24, 30, 36, 42 and 48 hours of growth in Super YEME supplemented with Tiger's milk. Aliquots of 10 µg protein were phosphorylated *in vitro*. *In vitro* phosphorylation reactions of 48-hour extracts were also supplemented with 10 mM $MnCl_2$ and 30 mM $CaCl_2$. Proteins were resolved on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and exposed to X-ray film at –70°C with an intensifying screen. Numbers at the right represent molecular weight markers (kDa).


A.
24
30
36
42
48
Mn
Ca
(kDa)
206
137
83
44.4
32.5
19
8.1
B.
24
30
36
42
48
Mn
Ca
(kDa)
206
137
83
44.4
32.5
19
8.1

Phosphorylation of J1501 (Plate III.2.1.1-A) CFE resulted in a temporal increase in the phosphorylation of an 80 kDa protein as well as a 68 kDa protein at 36 hours. By 36 hours in liquid culture, J1501 had started to produce a pink pigment corresponding to the red-pigmented antibiotic, undecylprodigiosin, which indicated the onset of chemical differentiation in liquid culture. By 48 hours, undecylprodigiosin was very noticeable in the cultures. In the presence of manganese, a phosphorylated protein at 120 kDa was observed which was not phosphorylated in the absence of manganese for the same time point; the same was observed for a phosphorylated protein with an apparent molecular weight of 88 kDa. Manganese did not appear to have an effect on the 80 or 68 kDa proteins. In the presence of calcium, a protein at 83 kDa was phosphorylated to a degree that was not observed in the presence or absence of manganese. The presence of calcium also seemed to decrease the amount of phosphorylation observed for the 32 kDa protein.

The phosphorylation profile of the *bldA*-deleted strain, J1681, revealed an apparent temporal increase (Plate III.2.1.1-B) in the phosphorylation of proteins at 120 kDa and 88 kDa. In comparison to the *bldA*$^+$ strain, an 80 kDa protein became more intensely phosphorylated at 30 hours as did a 68 kDa protein and a 53 kDa protein. In the presence of 10 mM $MnCl_2$, the 120 kDa protein became more phosphorylated than in the absence of manganese. The same was observed for the phosphorylated protein at 88 kDa. Manganese did not appear to have an effect on the 80 and 68 kDa protein. In the presence of 30 mM $CaCl_2$, an 83 kDa protein was phosphorylated to an extent not observed when manganese was present in the labelling reaction. The presence of calcium also decreased the amount of phosphorylation for the 32 kDa labelled band.

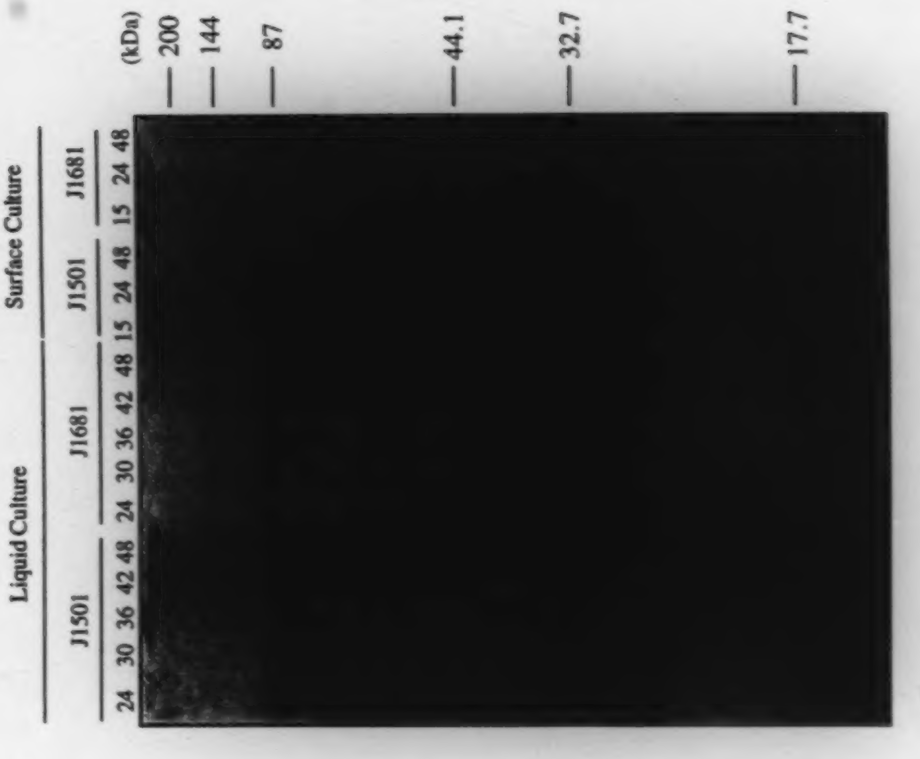
These liquid culture experiments indicated that there were proteins that were temporally phosphorylated. The appearance of some phosphorylated proteins seemed to occur later in the wild type compared with the *bldA*-deleted mutant. Interestingly, the phosphorylation of proteins seemed, overall, to be greater in the mutant strain than in the wild type strain. The phosphorylation profile was influenced by the presence of manganese and calcium. (This is addressed in greater detail in III.2.3).

The phosphorylation profiles of liquid culture extracts labelled by the method of Hong *et al.* (1993) were also investigated (Plate III.2.1.2). The major difference between the two methods was the temperature at which the *in vitro* reaction was allowed to proceed. A labelling temperature of 37°C was used in the Wang and Koshland (1981) protocol while the labelling temperature of the Hong *et al.* (1993) protocol was 25°C (room temperature). As was observed with the phosphorylation profile generated by the other labelling protocol (Wang and Koshland, 1981), there were relatively few phosphorylated proteins some of which were temporally phosphorylated. The 32 kDa protein appeared to be the major phosphorylated protein. The phosphorylation state of the 32 kDa protein appeared to be constant in liquid culture throughout growth for both J1501 and J1681.

III.2.2 Surface Culture

On a solid surface, *Streptomyces coelicolor* is able to undergo morphological as well as chemical differentiation, therefore, it was of interest to investigate the protein profiles obtained from surface cultures. Cell-free extracts were prepared at different

Plate III.2.1.2. Protein phosphorylation profiles for *Streptomyces coelicolor* J1501 (*bldA*$^+$) and J1681 (*bldA*$^-$) grown in liquid and surface cultures. Liquid culture time courses were carried out in Super YEME. Surface culture time courses were carried out on R2YE agar. Reaction products generated from *in vitro* labelling with [γ-^{32}P] ATP were separated on a 10% SDS-polyacrylamide gel. Proteins were transferred onto a PVDF membrane. Phosphorylated proteins were visualized by overnight exposure to a phosphorimager screen. Numbers at right represent molecular weight markers (kDa).


Liquid Culture
Surface Culture
J1501
J1681
J1501
J1681
24 30 36 42 48
24 30 36 42 48
15 24 48
15 24 48
(kDa)
200
144
87
44.1
32.7
17.7

times during the growth of *S. coelicolor* J1501 and J1681 as surface cultures on R2YE supplemented with Tiger's milk. As was observed with liquid cultures, total protein profiles of surface culture cell-free extracts were complex; *in vitro* protein phosphorylation was therefore investigated for surface grown cultures.

Surface culture cell-free extracts were labelled by the *in vitro* method of Hong *et al.* (1993). There was a significant difference in the phosphorylation profile of J1501 grown as surface cultures compared with J1681 grown in an identical manner (Plate III.2.1.2). In the higher molecular weight range there were more proteins phosphorylated in the wild type than in the mutant for the earlier time points (15 and 24 hours). The protein with an apparent molecular weight of 88 kDa was phosphorylated at 15 and 24 hours but was dephosphorylated by 48 hours, corresponding to the time when this strain had sporulated and produced antibiotics. The opposite was observed for J1681; the 88 kDa protein was not phosphorylated at 15 or 24 hours. Phosphorylation of this protein in the *bldA*-deletion strain occurred at 48 hours. This trend was also observed for a protein with an apparent molecular weight of 72 kDa. The phosphorylation of the protein with an apparent molecular weight of 32 kDa appeared to be more intense in the *bldA*-deletion strain compared to that of the wild type strain. The mutant strain, J1681, does not undergo morphological differentiation. To ensure that the observed phosphorylation profiles were not due to underloading the J1681 lanes the PVDF membrane was stained with Ponceau S. The 32 kDa protein band stained with the same intensity with Ponceau S for all the time points in both J1501 and J1681 (not shown). The 88 and 72 kDa bands were also present in the 15 and 24-hour samples at approximately the same intensity.

It was interesting that the two different *in vitro* phosphorylation protocols yielded slightly different profiles. The method by Hong *et al.* (1993) did not appear to be an efficient way to label proteins with an apparent molecular weight greater than 88 kDa. A comparison of the phosphorylation profiles between liquid and surface cultures revealed that in liquid culture, the temporal differences in phosphorylation were slight between the two strains, while phosphorylation profiles obtained from surface culture extracts showed a remarkably different temporal pattern with respect to the stage of growth in which a protein was phosphorylated or dephosphorylated. This may have been a reflection of the differences in growth between liquid and surface cultures; *Streptomyces coelicolor* does not undergo morphological differentiation in liquid culture.

There were temporal differences observed in phosphorylation profiles between the mutant and the wild type strain for both liquid and surface cultures indicating that the use of phosphorylation as a means to identify *bldA* targets would be a viable approach.

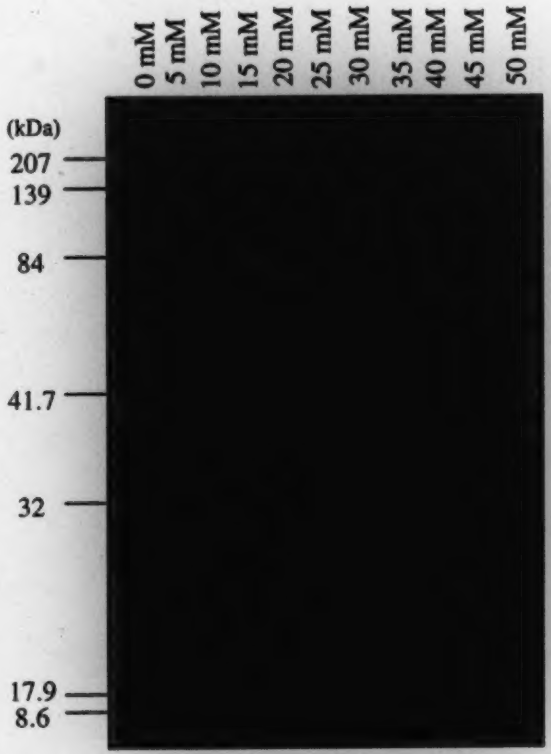
III.2.3 Effect of Mn^{2+} and Ca^{2+} on Phosphorylated Protein Profiles

Since it was observed that manganese and calcium, when supplied in the concentration determined to be optimal for *in vitro* labelling of S. *griseus* proteins (Hong *et al.*, 1993), had an effect on the phosphorylation profiles of J1501 and J1681 (see Plate III.2.1.1) it was of interest to determine the optimal concentrations for *S. coelicolor*. *In vitro* phosphorylation reactions of 48-hour surface culture whole cell-free extracts (Hong *et al.*, 1993) were supplemented with increasing concentrations of $MnCl_2$ and $CaCl_2$ in the range of 0 mM to 50 mM (Plate III.2.3.1). The 48-hour CFE was chosen because by this time, surface cultures were differentiated and also that was the time point when the most phosphorylated proteins became apparent. In the absence of manganese in the *in vitro* labelling reaction of 48-hour J1501 surface culture CFEs (Plate III.2.3.1-A), there were three protein bands that showed significant phosphorylation: one protein band, with an apparent molecular weight of 68 kDa, one with an apparent molecular weight of 32 kDa, and a small protein at ~17.9 kDa. The phosphorylation intensity of the 68 kDa protein remained relatively constant until concentrations of $MnCl_2$ greater than 30 mM were added. With concentrations greater than 30 mM, the phosphorylation intensity decreased. This trend was also observed for the 32 kDa-phosphorylated protein. The 32 kDa protein appeared to be maximally phosphorylated with 20 mM manganese added to the phosphorylation reaction..

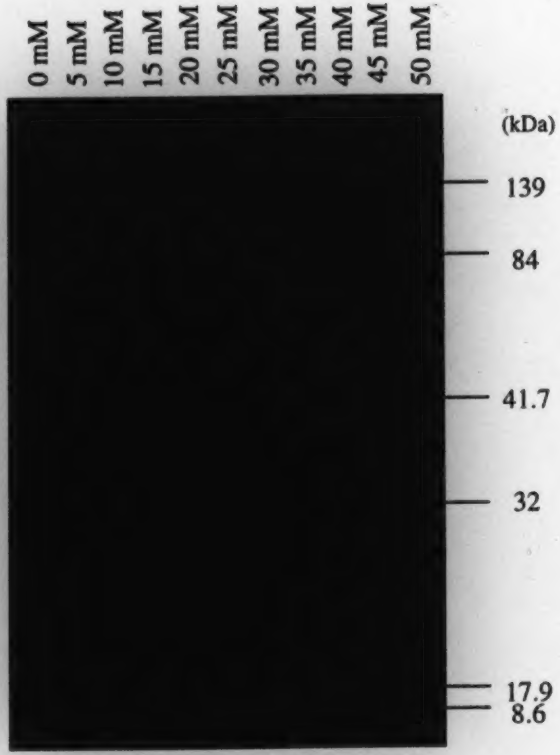
When 48-hour CFEs of J1681 were labelled in a similar manner (Plate III.2.3.1-B), there was a greater number of phosphorylated proteins overall. In J1681 labelled cell-free extracts, there were four protein bands that were significantly phosphorylated. A phosphorylated protein with an apparent molecular weight of 120 kDa which was observed in the absence of manganese (as well as in the reactions with increasing concentrations of manganese in J1681) was not phosphorylated in J1501 in the absence of manganese. When manganese was present in the *in vitro* phosphorylation reactions, the 32 kDa protein was phosphorylated to about the same extent for the entire range of manganese concentrations tested compared to the same protein in the wild type strain. In J1501, the phosphorylation of the 32 kDa protein decreased with concentrations greater than 30 mM $MnCl_2$. Two additional phosphorylated bands were observed with a concentration of 10 mM $MnCl_2$ than were observed with the other concentrations

Plate III.2.3.1. Effect of $MnCl_2$ concentration on the phosphorylation profiles of *Streptomyces coelicolor* J1501 (A) and J1681 (B). The 48-hour surface culture cell-free extracts were supplemented with concentrations of $MnCl_2$ from 0 mM to 50 mM in the *in vitro* phosphorylation reactions. Aliquots containing 10 µg protein were resolved on 10% SDS-polyacrylamide gels, transferred to nitrocellulose and autoradiographed at –70°C with an intensifying screen for 3 days. Molecular weights (in kDa) are shown to the left and right of panels A and B respectively.

A.


0 mM
5 mM
10 mM
15 mM
20 mM
25 mM
30 mM
35 mM
40 mM
45 mM
50 mM
(kDa)
207
139
84
41.7
32
17.9
8.6

B.

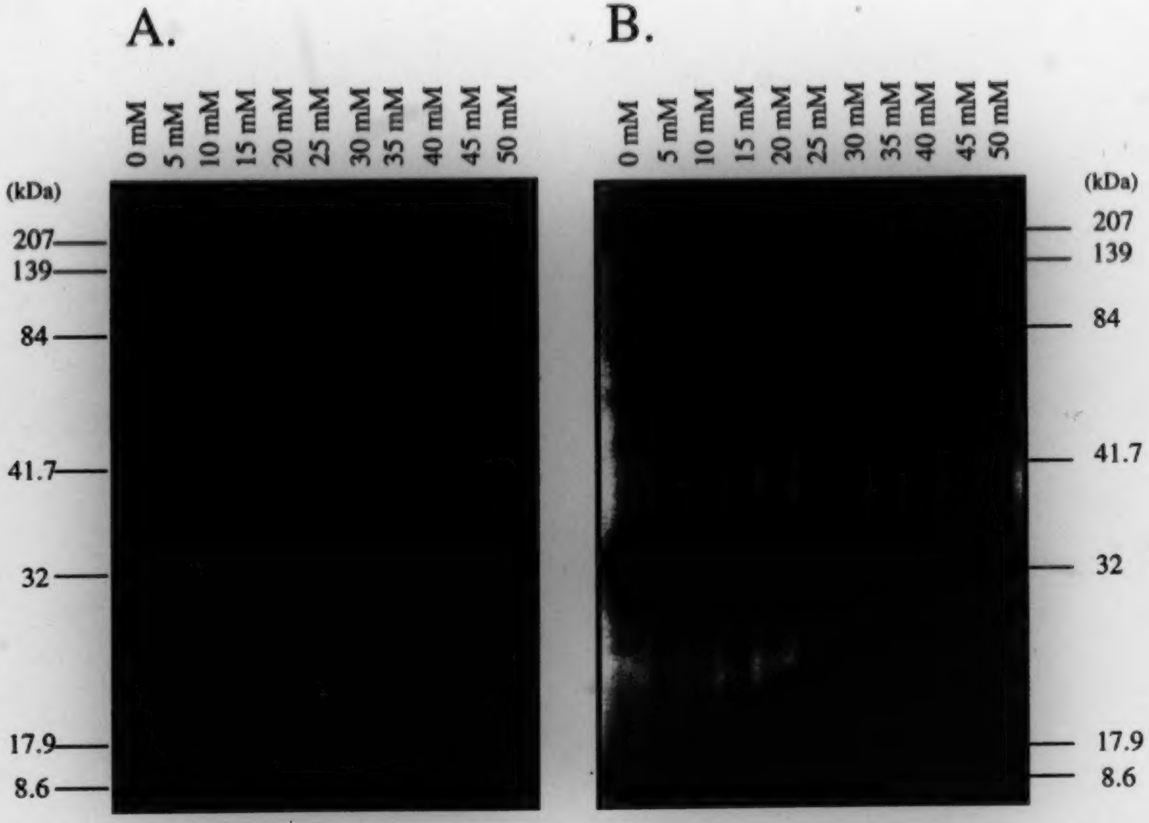

0 mM
5 mM
10 mM
15 mM
20 mM
25 mM
30 mM
35 mM
40 mM
45 mM
50 mM
(kDa)
139
84
41.7
32
17.9
8.6

tested. This was also the concentration Hong *et al.* (1993) reported for phosphorylation of proteins from *S. griseus.*

In vitro phosphorylation reactions of 48-hour surface culture cell-free extracts supplemented with increasing concentrations of $CaCl_2$ are shown in Plate III.2.3.2. *S. coelicolor* J1501 48-hour extracts (Plate III.2.3.2-A) without the addition of calcium, possessed three major phosphorylated protein bands. The 68 kDa-phosphorylated protein was only significantly phosphorylated in the absence of calcium. (Recall that this protein was phosphorylated in the presence of manganese). Addition of calcium to *in vitro* labelling reactions did not have an effect on the phosphorylation of the 32 kDa protein for 48-hour extracts of J1501. *S. coelicolor* J1681 48-hour extracts (Plate III.2.3.2-B), in the absence of additional calcium in labelling reactions, possessed four major phosphorylated protein bands. As was observed for labelling reactions supplemented with manganese, J1681 possessed a greater number of phosphorylated proteins than did the wild type strain, J1501. Interestingly, the 32 kDa protein was phosphorylated to a greater extent in the *bldA*-deleted strain than in the wild type when calcium was used to supplement the phosphorylation reactions. In fact, the 32 kDa protein was more intensely phosphorylated in J1681 regardless of whether manganese, calcium or no such additions were made to the phosphorylation reactions.

The optimum labelling of proteins with [γ-^{32}P] ATP observed with the addition of $CaCl_2$ was achieved at a concentration of 30 mM. At this concentration, the

Plate III.2.3.2. Effect of $CaCl_2$ concentration on the phosphorylation profiles of *Streptomyces coelicolor* J1501 (A) and J1681 (B). The 48-hour surface culture cell-free extracts were supplemented with concentrations of $CaCl_2$ from 0 mM to 50 mM in the *in vitro* phosphorylation reactions. Aliquots containing 10 µg protein were resolved on 10% SDS-polyacrylamide gels, transferred to nitrocellulose and autoradiographed at –70°C with an intensifying screen for 3 days. Molecular weights (in kDa) are shown to the left and right of panels A and B respectively.


A.
B.
0 mM
5 mM
10 mM
15 mM
20 mM
25 mM
30 mM
35 mM
40 mM
45 mM
50 mM
(kDa)
207
139
84
41.7
32
17.9
8.6

greatest number of phosphorylated proteins was observed. This is in agreement with the concentration reported for phosphorylation of proteins from *S. griseus* (Hong *et al.*, 1993).

Manganese and calcium were both found to have an effect on the phosphorylation profiles of J1501 and J1681. The mutant strain, J1681, showed better labelling of proteins overall under any reaction conditions. In the presence of calcium and manganese, higher molecular weight proteins were labelled with the procedure of Hong *et al.* (1993) that were not labelled without their addition. This labelling procedure was adopted and routinely used because it had been established for phosphorylation in *Streptomyces*, and therefore use of the same labelling procedure would enable a more accurate comparison of phosphorylation with other *Streptomyces* species.

One-dimensional SDS-polyacrylamide gel analysis revealed the presence of a phosphorylated 32 kDa band that showed significantly higher phosphorylation in the *bldA*$^-$ strain suggesting that *bldA* controls the phosphorylation state of this protein. Research efforts were focussed on optimizing the resolution of the 32 kDa-phosphorylated protein so that it could be purified and characterized.

III.2.4 Effect of DNAse I and RNAse A Digestion on Protein Profiles

It was observed with two-dimensional gel electrophoresis of whole cell-free extracts, that fewer high-molecular weight phosphorylated proteins were present compared to the number observed with one-dimensional gel electrophoresis. Samples for two-dimensional (2-D) gel analysis were treated with DNase I and RNase A since this treatment reduced streaking in the first (isoelectric focusing) dimension. It was also found to eliminate some of the higher molecular weight proteins. Since it is was possible that some of these higher molecular weight bands were degraded to yield some of the lower molecular weight bands observed, additional protease inhibitors were added during the extraction process and to the sample buffer. A series of experiments was performed with unlabelled proteins in an effort to determine if some of the higher molecular weight bands were a result of nucleic acid present in the samples or if the higher molecular weight proteins were degraded by proteases that may have been present in the DNase I mixture. Plate III.2.4.1 shows protein profiles of 10 µg aliquots of liquid culture whole cell-free extracts without treatment with DNase I/RNase A and with digestion with both of these enzymes in combination. Digestion of proteins with DNase I/RNase A was found to alter the protein profile. Some of the high molecular weight bands disappeared and lower molecular weight bands became apparent.

Since DNase I may have been contaminated with proteases that degrade susceptible proteins, phenylmethylsulfonyl fluoride (PMSF) at 1 mM was added to aliquots of DNase I and RNase A and 10 µg protein samples were digested as described for two-dimensional gel analysis. No significant difference (Plate III.2.4.2) was observed between reactions that contained the protease inhibitor, PMSF, and those that did not, indicating that either degradation of undigested extracts was not responsible for the loss of the higher molecular weight proteins or that PMSF was not a suitable protease inhibitor. Manganese and calcium were added to separate digestion reactions to determine if they had any effect on digestion profiles with DNaseI or RNase A (Plate

Plate III.2.4.1. Effect of DNaseI/RNaseA digestion on protein profiles of *Streptomyces coelicolor* J1501 (*bldA*$^+$) and J1681 (*bldA*$^-$). Protein profiles of liquid culture cell-free extracts from time course experiments were compared to protein profiles resulting from digestion with DNaseI/RNaseA on silver stained 12% SDS-polyacrylamide gels. Aliquots of 48-hour samples containing 10 mM $MnCl_2$ and 30 mM $CaCl_2$ were also digested and compared to identical samples without DNaseI/RNaseA digestion.

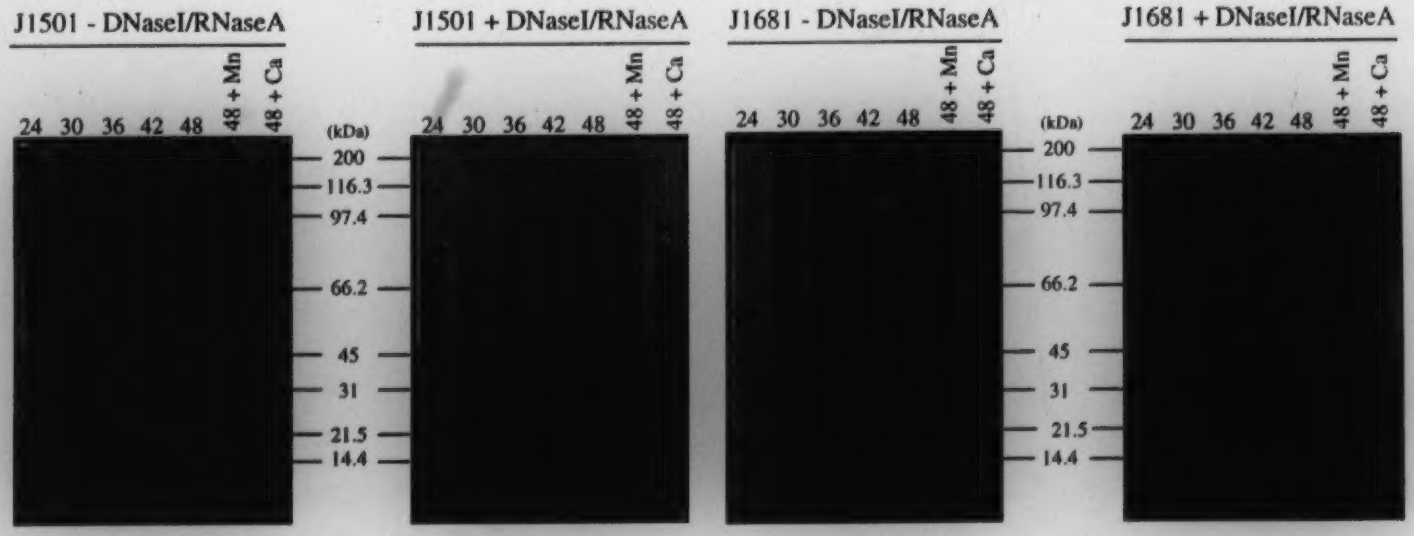
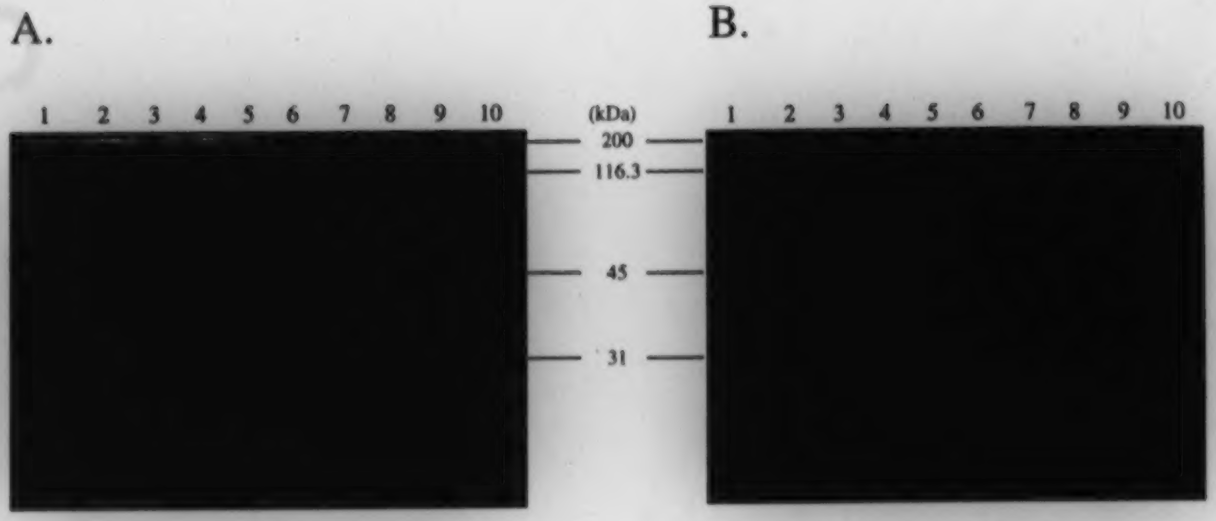

J1501 - DNaseI/RNaseA
24
30
36
42
48
48 + Mn
48 + Ca
(kDa)
200
116.3
97.4
66.2
45
31
21.5
14.4
J1501 + DNaseI/RNaseA
24
30
36
42
48
48 + Mn
48 + Ca
J1681 - DNaseI/RNaseA
24
30
36
42
48
48 + Mn
48 + Ca
(kDa)
200
116.3
97.4
66.2
45
31
21.5
14.4
J1681 + DNaseI/RNaseA
24
30
36
42
48
48 + Mn
48 + Ca

Plate III.2.4.2. Comparison of protein profiles of liquid culture extracts of ***Streptomyces coelicolor*** (A) J1501 (***bldA***$^+$) and (B) J1681 (***bldA***$^-$) supplemented with protease inhibitor and digested with DNaseI/RNaseA. Cell-free extracts from 48-hour samples were subjected to digestion in the presence of phenylmethylsulfonyl fluoride (PMSF) and electrophoresed on 12% SDS-PAGs. Proteins were visualized by silver staining. (1) Untreated CFE, (2) CFE supplemented with 1 mM PMSF, (3) CFE digested with DNaseI/RNaseA, (4) CFE supplemented with 1 mM PMSF and digested with DNaseI/RNaseA, (5) CFE digested with DNaseI, (6) CFE supplemented with 1 mM PMSF and digested with DNaseI, (7) CFE digested with RNaseA, (8) CFE supplemented with 1 mM PMSF and digested with RNaseA, (9) CFE supplemented with 10 mM $MnCl_2$, (10) CFE supplemented with 30 mM $CaCl_2$.


A.
B.
1
2
3
4
5
6
7
8
9
10
(kDa)
200
116.3
45
31

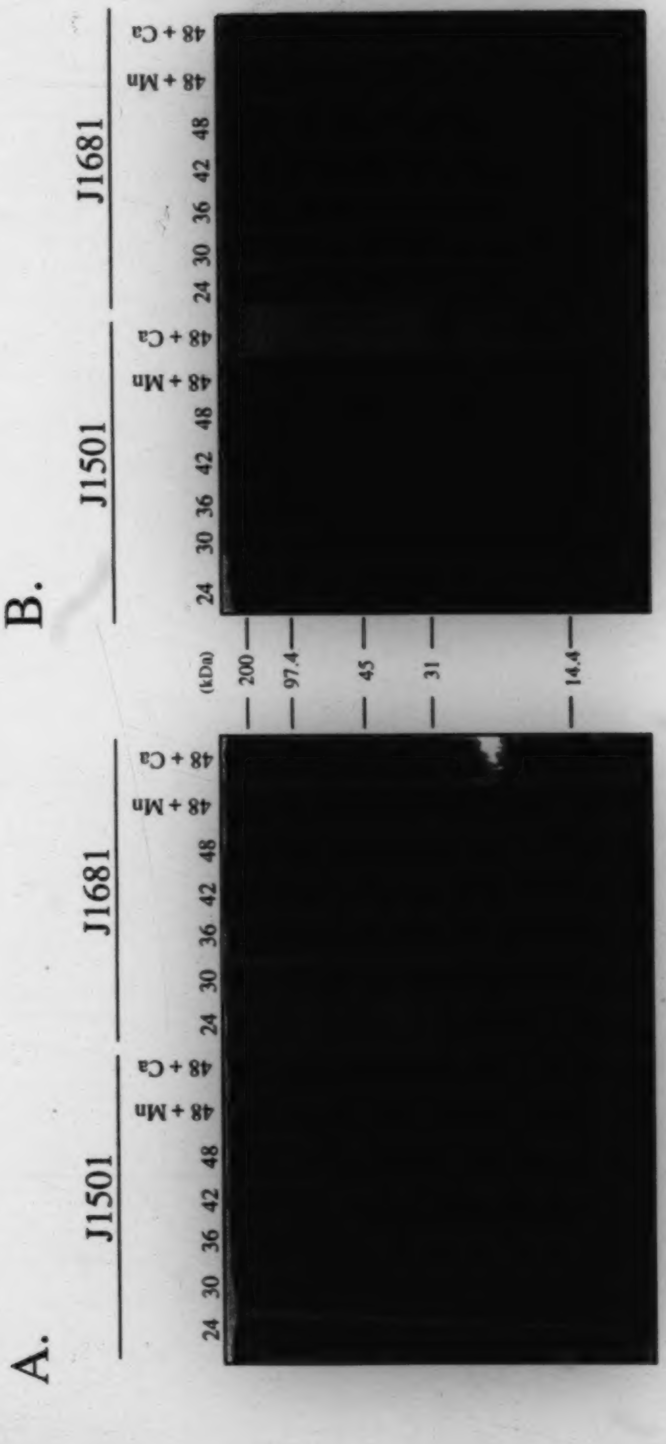
III.2.4.1 and Plate III.2.4.2). Neither manganese nor calcium had a significant effect on the digestion profiles of proteins in these investigations.

Protein profiles of J1501 and J1681 were compared upon treatment with DNase I alone supplemented with PMSF, and RNase A alone supplemented with PMSF over a time course in liquid culture (Plate III.2.4.3). It was found that a significant number of lower molecular weight bands were generated upon digestion with DNase I supplemented with PMSF (Plate III.2.4.3-A) compared with RNase A supplemented with PMSF (Plate III.2.4.3-B). In light of these results, additional protease inhibitors (Pefabloc, which is an irreversible serine protease inhibitor and Pepstatin A which inhibits aspartic proteases) were added to cell-free extracts during the extraction procedure as well as prior to storage at -70°C.

III.3 Comparison of Protein Profiles By Two-Dimensional Gel Electrophoresis

Since protein profiles of J1501 (*bldA*$^+$) and J1681 (*bldA*$^-$) were found to be very complex and unlikely to allow sufficient resolution of the phosphorylated 32 kDa band for purification, two-dimensional (2-D) gels were used to obtain a better resolution of proteins since this method can be used to separate thousands of proteins (O'Farrell, 1975), (Garrels, 1989). This technique combines isoelectric focusing (IEF), which separates proteins in a mixture according to charge (isoelectric point; pI), with the size separation of SDS-polyacrylamide gel electrophoresis. Comparative analysis of high-resolution 2-D gels of cell-free extracts from a *bldA*$^+$ and a *bldA*$^-$ strain were used in an attempt to identify potential *bldA* targets. The protein samples for 2-D electrophoresis were treated the same as for one dimensional electrophoresis except that the samples for 2-D work

Plate III.2.4.3. Comparison of liquid culture protein profiles of *Streptomyces coelicolor* J1501 (*bldA*$^+$) and J1681 (*bldA*$^-$) generated by digestion with DNaseI and RNaseA. Aliquots of protein from various stages of growth in Super YEME supplemented with Tiger's milk were digested with either (A) DNaseI containing 1 mM PMSF or (B) RNaseA containing 1 mM PMSF. The effect of 10 mM $MnCl_2$ and 30 mM $CaCl_2$ on protein profiles of 48-hour cell-free extracts was also assessed. Proteins were resolved on 12% SDS-PAGs and silver stained.


A.
B.
J1501
J1681
24
30
36
42
48
48 + Mn
48 + Ca
(kDa)
200
97.4
45
31
14.4

were digested with DNase I (1 mg/mL) and RNase A (0.25 mg/mL). It was found that this treatment greatly reduced sample streaking in the isoelectric focusing step in the first dimension.

To optimize the amount of protein to load on two-dimensional gels, aliquots containing 10, 25, 50, 100, 150, 200 and 300 µg of protein (Plate III.3.1 and Plate III.3.2) from 48-hour J1501 liquid culture extracts were isoelectric focused and subjected to SDS-polyacrylamide gel electrophoresis as described (II.3.5). An increase in the amount of protein resulted in a greater number of spots observed. When greater than 50 µg protein was analyzed, the size of some of the spots increased thereby decreasing the distance between neighboring spots with a concomitant loss of resolution, therefore, a lower protein load was optimal since it resulted in the resolution of a greater number of protein spots. Since the less protein applied onto the first dimension, the better the resolution, 10-25 µg of protein were routinely used for 2-D gel analysis.

It was found that reproducibility of two-dimensional gels was a serious problem making comparisons between gels difficult. Although this problem is not uncommon (Garrels, 1989), attempts were made to optimize conditions for 2-D gels. Parameters that were investigated included sample buffer detergents, isoelectric focusing times and ampholyte concentration used. Because of the reproducibility problems, IEF gels were run in triplicate per sample in order to obtain at least two reproducible two-dimensional profiles per sample (Plate III.3.3); this was not always achieved (Plate III.3.4). The 2-D

Plate III.3.1. Effect of protein load on two-dimensional gel analysis. Aliquots of (A) 10 µg, (B) 25 µg, (C) 50 µg, and (D) 100 µg protein from *Streptomyces coelicolor* J1501 liquid culture cell-free extracts from 48-hour samples were subjected to two-dimensional gel analysis. The pH range for isoelectric focusing (IEF) was pH 3 to pH 10 expanded in the pH 5 to pH 7 range. Electrophoresis in the second dimension was carried out using 12.5% SDS-PAGE.

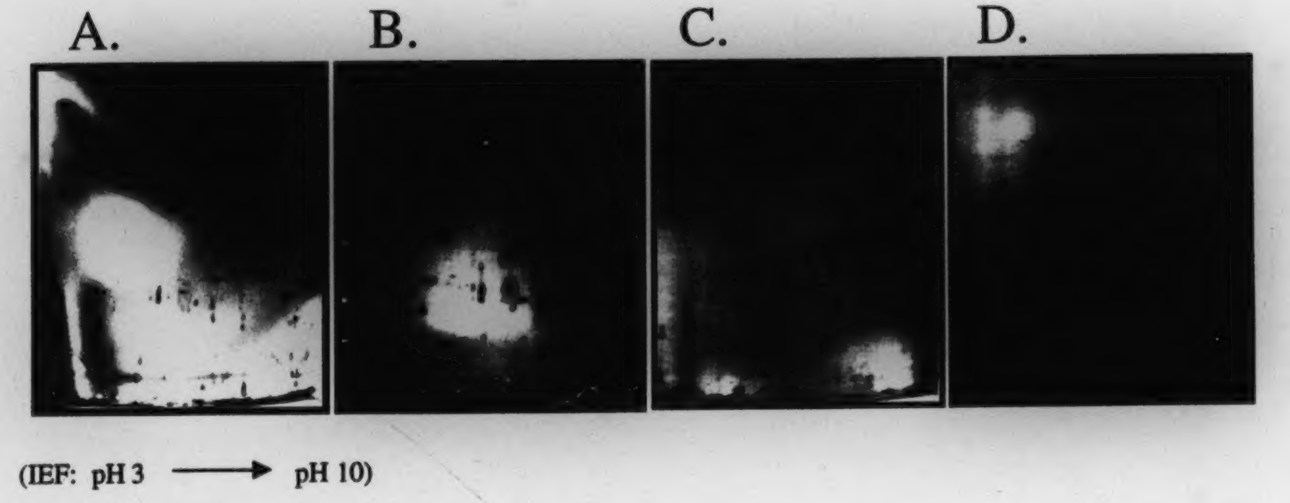

A.
B.
C.
D.
(IEF: pH 3 ⟶ pH 10)

Plate III.3.2. Two-dimensional gel analysis of proteins extracted from *Streptomyces coelicolor* J1501 grown in liquid culture. Liquid culture cell-free extracts of 48-hour samples containing (A) 150 µg, (B) 200 µg, and (C) 300 µg of protein were subjected to isoelectric focusing and then electrophoresed in the second dimension using 12.5% SDS-PAGE and silver stained. The range of pH for isoelectric focusing (IEF) was pH 3 to pH 10. The pH gradient was expanded in the pH 5 to pH 7 range.

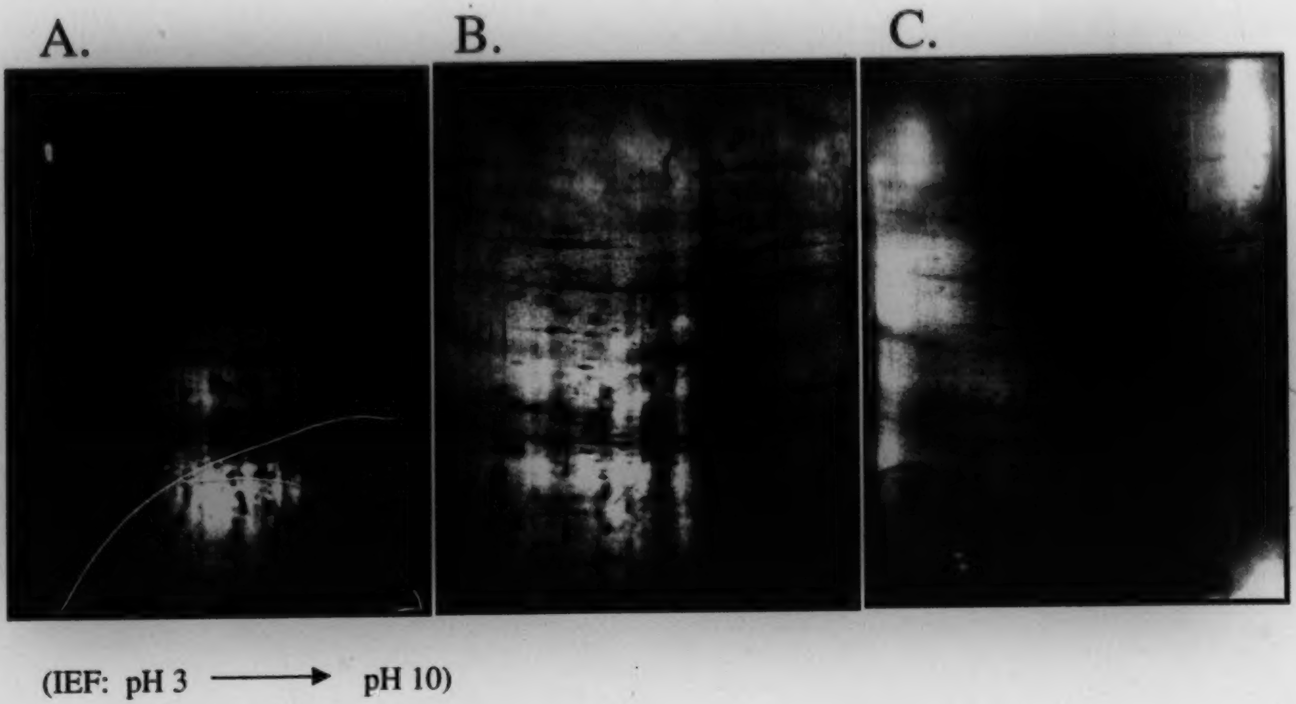

A.
B.
C.
(IEF: pH 3 ⟶ pH 10)

Plate III.3.3. Two-dimensional gel analyses of proteins extracted from *Streptomyces coelicolor* J1681 grown in liquid culture. Aliquots of 25 µg protein from 48-hour cultures of *S. coelicolor* J1681 grown in Super YEME supplemented with Tiger's milk were, in triplicate, subjected to isoelectric focusing, electrophoresis on 12.5% SDS-PAGs and silver stained. The range of pH for isoelectric focusing (IEF) was pH 3 to pH 10 expanded in the pH 5 to 7 range.

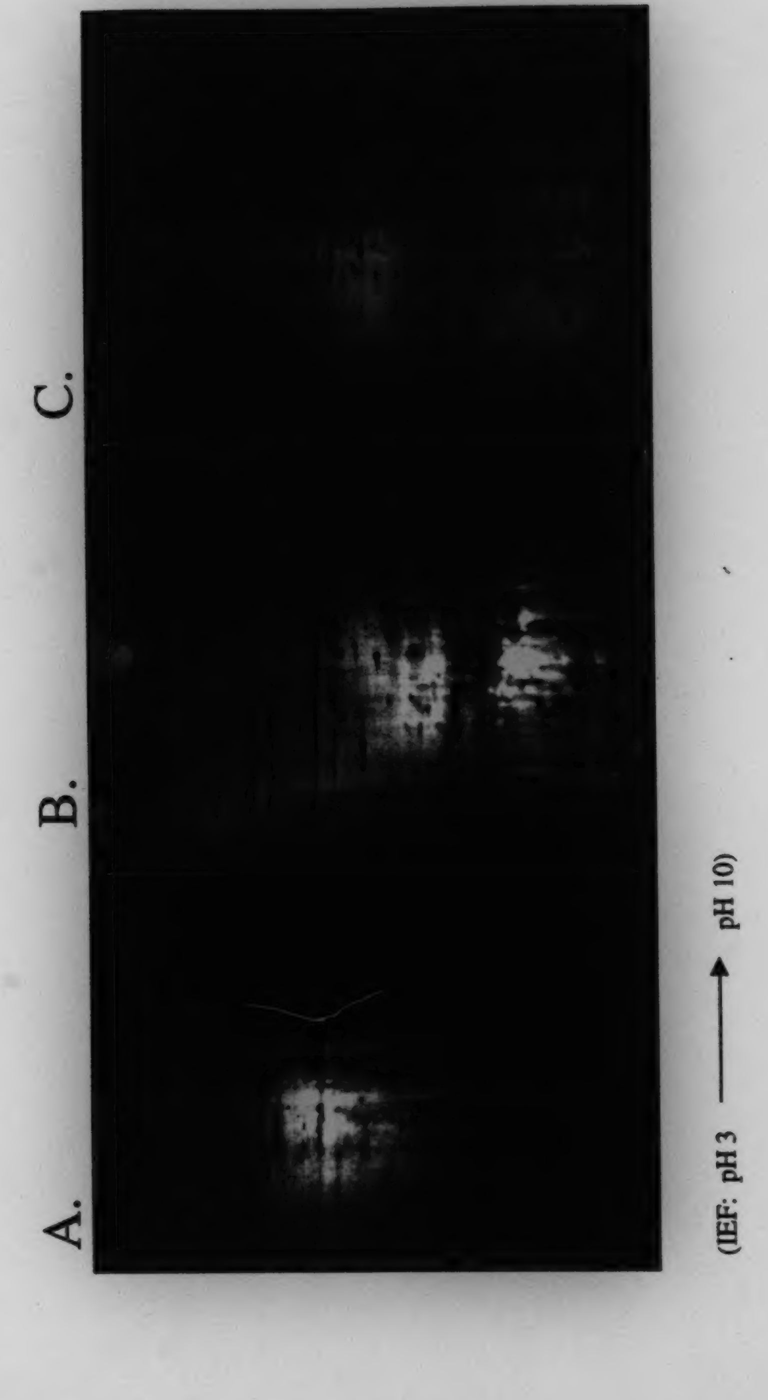
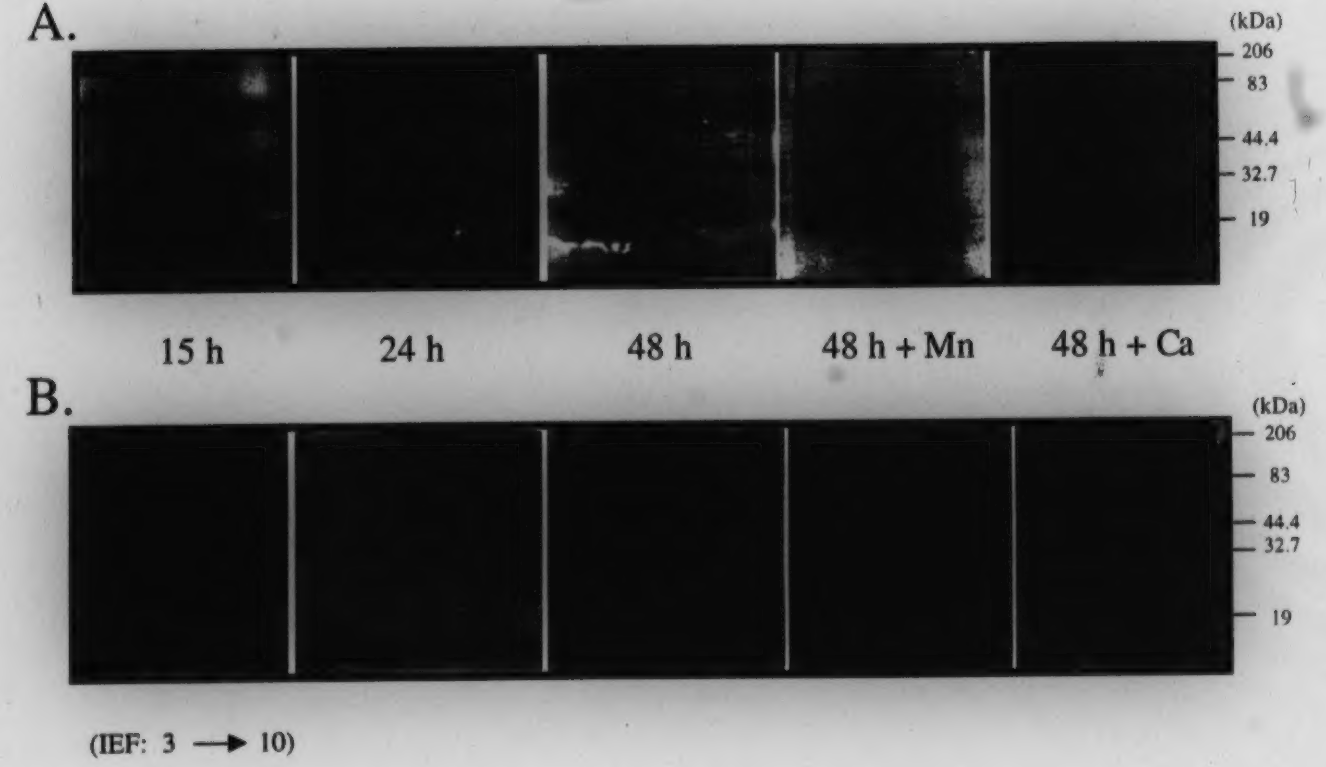

A.
B.
C.
(IEF: pH 3 → pH 10)

Plate III.3.4. Two-dimensional gel analysis of proteins expressed during the growth of *Streptomyces coelicolor* on surface cultures. *S. coelicolor* J1501 (A) and J1681 (B) were grown on the surface of R2YE agar supplemented with Tiger's milk and cell-free extracts were prepared from 15, 24, and 48 hours post-inoculum. Protein extracted at 48-hours was also supplemented with 10 mM $MnCl_2$ or 30 mM $CaCl_2$. Profiles generated by two-dimensional electrophoresis were visualized by silver staining. The pH range for isoelectric focusing (IEF) was pH 3 to 10 expanded in the pH 5 to 7 range. Molecular weights (kDa) are shown.

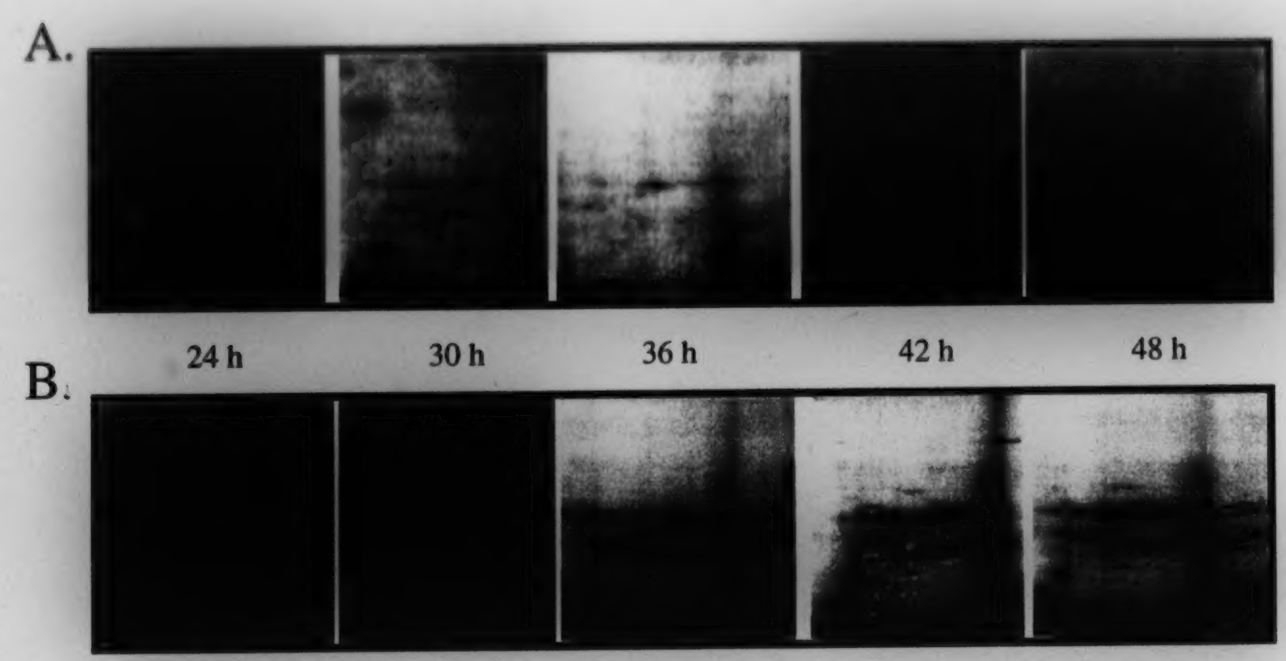

A.
(kDa)
206
83
44.4
32.7
19
15 h
24 h
48 h
48 h + Mn
48 h + Ca
B.
(kDa)
206
83
44.4
32.7
19
(IEF: 3 ⟶ 10)

protein profiles of total protein extracts were complex (Plate III.3.3), making reproducibility between gels extremely important. Two-dimensional gels (Plate III.3.4) of cell-free extracts from surface cultures of J1501 and J1681 illustrate the problems encountered with irreproducibility between gels. Since most of the protein spots were missing from the J1681 profiles, it may appear that there were significantly less proteins expressed in this strain compared with the wild type strain; this was known not to be the case (Plate III.2.1.1; Plate III.2.2.1). Protein failing to enter the IEF tube gels was likely responsible for these poor profiles. On occasion, streaking in the first dimension (IEF) was evident even though samples were digested with DNase I and RNase A.

Despite difficulties encountered with two-dimensional analyses of unlabelled extracts, attempts were made at trying to resolve phosphorylated proteins. In Plate III.3.5 the autoradiograms of the two-dimensional analyses of aliquots of 10 μg protein extracted from liquid culture time courses of J1501 and J1681 which had been labelled by the method of Wang and Koshland (1981) are shown. Two-dimensional gels of phosphorylated extracts of J1681 appeared to have a greater number of phosphorylated proteins. This was in agreement with the one-dimensional gel analysis of phosphorylated protein profiles. Due to the problems experienced with 2-D gel analysis, quantification of the spots, although attempted, was not pursued because irreproducibility of the gels made comparisons suspect.

Since one-dimensional gels had shown a difference in phosphorylation profiles between the two labelling methods described, 10 μg-aliquots of liquid culture cell-free extracts of J1501 and J1681 were labelled by the method of Hong *et al.* (1993) and analyzed by 2-D gel electrophoresis. Phosphorylated protein spots appeared to be

Plate III.3.5. Two-dimensional gel analysis of proteins phosphorylated during growth of *Streptomyces coelicolor* in liquid culture. *S. coelicolor* (A) J1501 and (B) J1681 were grown in Super YEME supplemented with Tiger's milk and protein was extracted at various times post-inoculation. Proteins were phosphorylated *in vitro* with [γ-^{32}P] ATP and subjected to two-dimensional gel analysis, transferred to nitrocellulose membranes and exposed to X-ray film with an intensifying screen at –70°C for 4 days. The pH range for isoelectric focusing (IEF) was pH 3 to pH 10 expanded in the pH 5 to pH 7 range.


A.
B.
24 h
30 h
36 h
42 h
48 h

(IEF: 3 ⟶ 10)

more detectable with this method (Plate III.3.6). There were several phosphorylated protein spots with an apparent molecular weight of 32 kDa which differred in isoelectric point (pI).

Because it had been noted that the labelling method of Hong *et al.* (1993) resulted in a more efficient labelling of phosphorylated proteins, it was of interest to investigate whether this was due to the labelling buffer or the temperature at which labelling was performed. The major difference between the labelling buffers between the two protocols is the addition of EDTA in the 'Hong' buffer. For the sake of optimizing the phosphorylation reactions, the labelling temperature of this method was used with the buffer of Wang and Koshland (1981) to determine if the buffer composition influenced the labelling of proteins. Western blots of the two-dimensional gels in which 10 μg of protein from cell-free extracts of 48-hour liquid cultures were labelled with 10 mM $MnCl_2 \cdot 4H_2O$ in 'Hong' labelling buffer (Hong *et al.*, 1993) and in 'Wang' labelling buffer (Wang and Koshland, 1981) were prepared. The labelling buffer did appear to have an effect on the labelling of phosphorylated proteins with [γ-^{32}P] ATP (Plate III.3.7). Labelling reactions with the 'Hong' buffer resulted in a more intensely phosphorylated protein profile than did the 'Wang' buffer. The nitrocellulose membranes were stained with India ink (Plate III.3.8) to visualize the total protein profiles of these two-dimensional gels so that profile comparisons could be made since it was important to determine if differences in phosphorylation profiles were due to gel irreproducibility or to significant protein differences. *In vitro* labelling reactions were more efficient when the 'Hong' labelling buffer was used.

Plate III.3.6. Comparison of proteins phosphorylated by ATP in liquid culture cell-free extracts as analyzed by two-dimensional gel electrophoresis. *Streptomyces coelicolor* (A) J1501 and (B) J1681 liquid culture cell-free extracts from cultures grown in Super YEME supplemented with Tiger's milk were phosphorylated *in vitro* with [γ-^{32}P] ATP and subjected to 2-D gel electrophoresis. Proteins were transferred to nitrocellulose membranes and phosphorylated proteins visualized by exposure to X-ray film with intensifying screen at –70°C for 2 days. The pH range for isoelectric focusing (IEF) was pH 3 to pH 10 expanded in the pH 5 to pH 7 range. Gels were aligned on the basis of molecular weight markers.

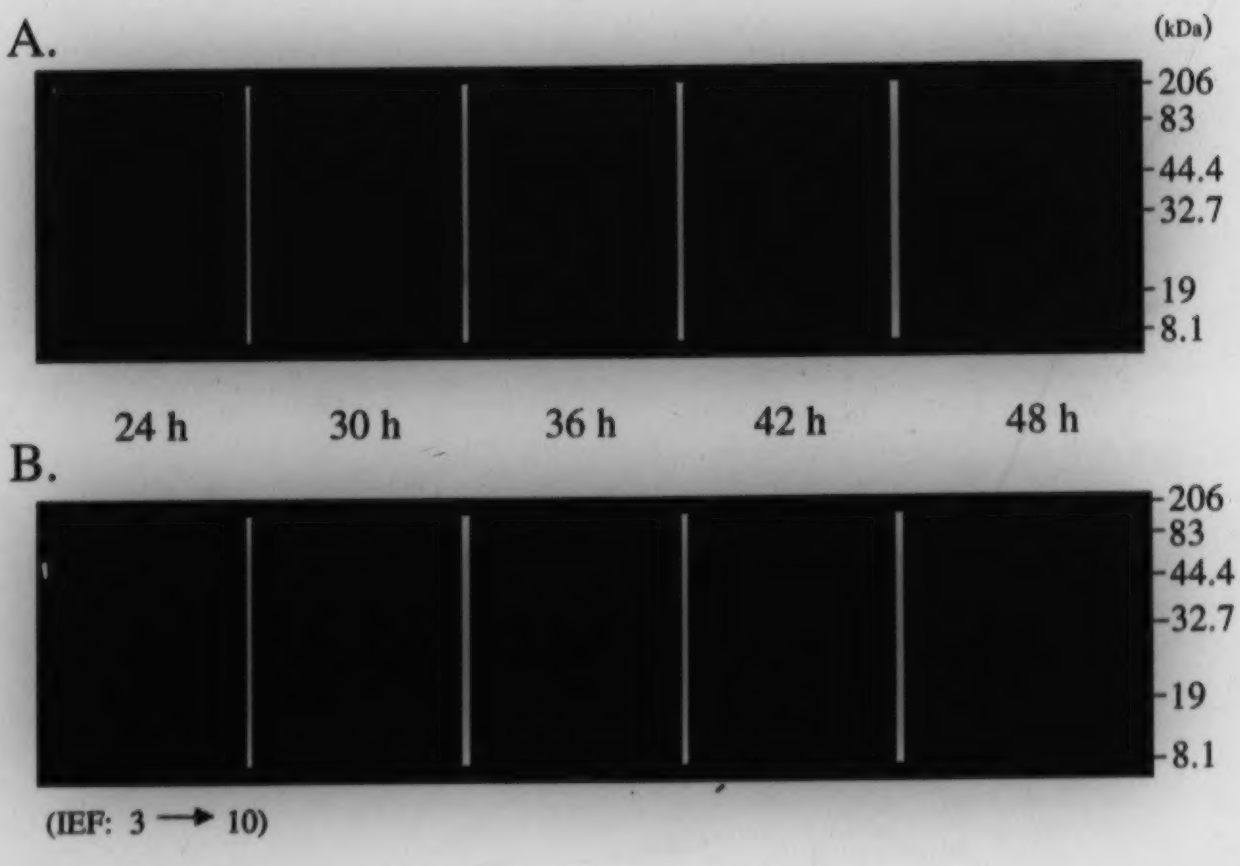

A.
(kDa)
206
83
44.4
32.7
19
8.1
24 h
30 h
36 h
42 h
48 h
B.
206
83
44.4
32.7
19
8.1
(IEF: 3 ⟶ 10)

Plate III.3.7. Characterization of proteins phosphorylated in the presence of $MnCl_2$ by two-dimensional gel electrophoresis. Proteins extracted from 48-hour liquid cultures of J1501 and J1681 were phosphorylated *in vitro* in reactions supplemented with 10 mM $MnCl_2$ in either 'Hong' labelling buffer or 'Wang' labelling buffer. (A) J1501 in 'Hong' labelling buffer (B) J1681 in 'Hong' labelling buffer (C) J1501 in 'Wang' labelling buffer (D) J1681 in 'Wang' labelling buffer. Proteins were subjected to 2-D gel analysis, transferred to nitrocellulose membranes, and autoradiographed for 2 days at -70°C with an intensifying screen. The pH range for isoelectric focusing (IEF) was pH 3 to pH 10 expanded in the pH 5 to pH 7 range. Molecular weights (in kDa) are shown.

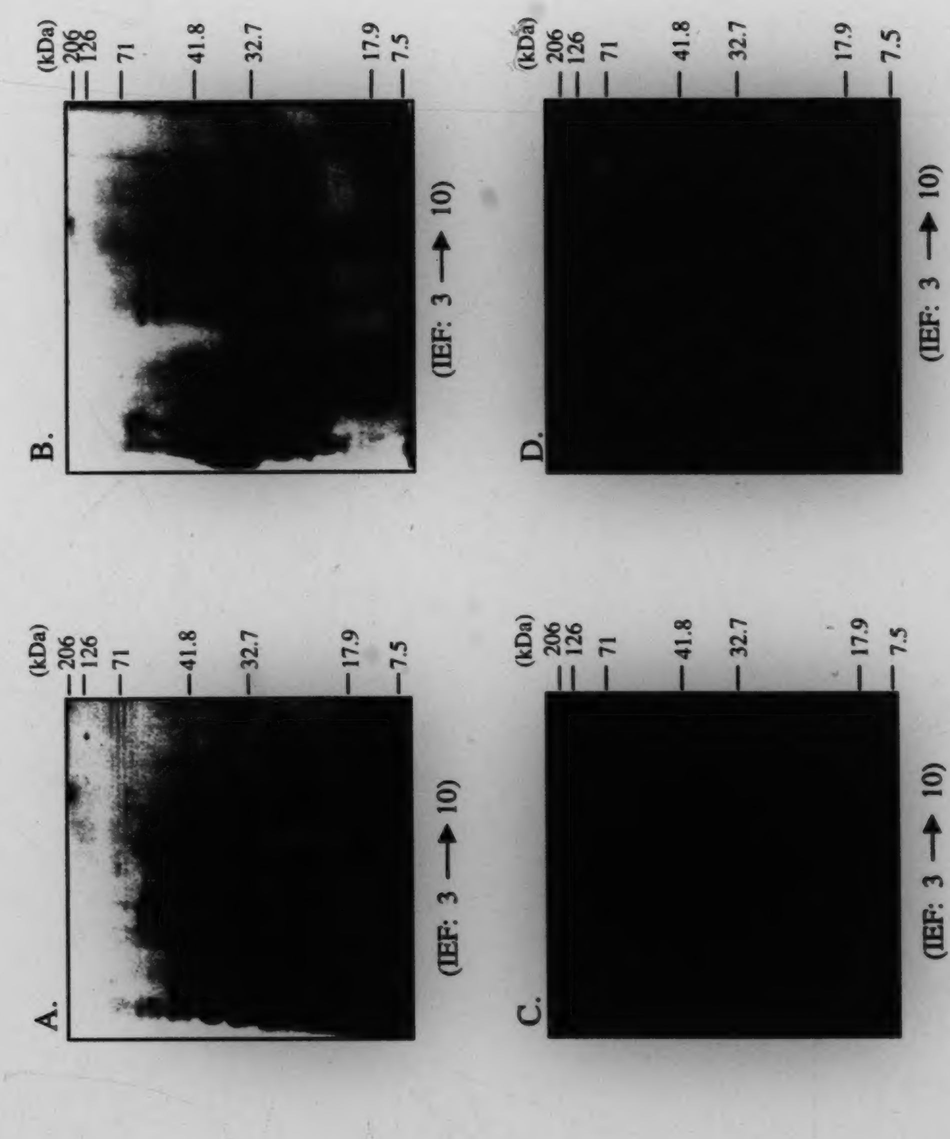
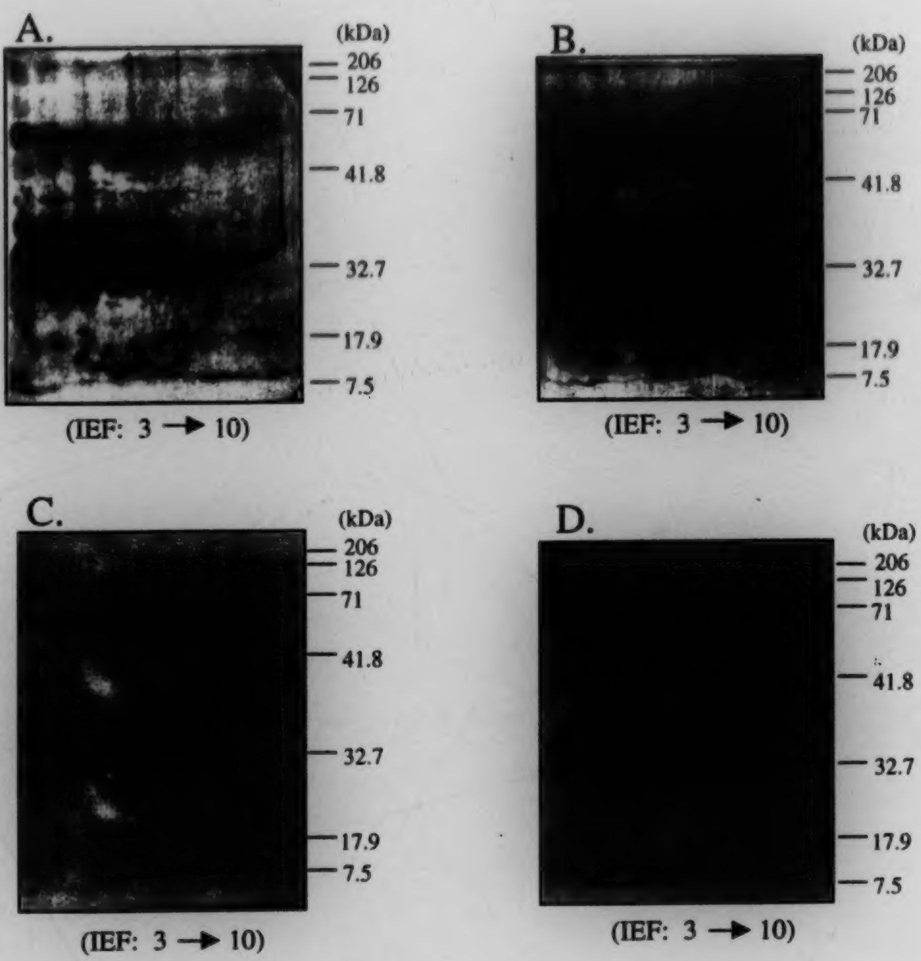

A.
B.
C.
D.
(kDa)
206
126
71
41.8
32.7
17.9
7.5
(IEF: 3 → 10)

Plate III.3.8. India ink stained nitrocellulose membranes of two-dimensional gels of proteins phosphorylated in the presence of 10 mM $MnCl_2$. (A) J1501 in 'Hong' labelling buffer (B) J1681 in 'Hong' labelling buffer (C) J1501 in 'Wang' labelling buffer (D) J1681 in 'Wang' labelling buffer. Proteins were subjected to 2-D gel analysis and transferred to nitrocellulose membranes. The pH range for isoelectric focusing (IEF) was pH 3 to pH 10 expanded in the pH 5 to pH 7 range. Molecular weights (in kDa) are shown.


A.
(kDa)
206
126
71
41.8
32.7
17.9
7.5
(IEF: 3 → 10)
B.
(kDa)
206
126
71
41.8
32.7
17.9
7.5
(IEF: 3 → 10)
C.
(kDa)
206
126
71
41.8
32.7
17.9
7.5
(IEF: 3 → 10)
D.
(kDa)
206
126
71
41.8
32.7
17.9
7.5
(IEF: 3 → 10)

The two-dimensional characterization of phosphorylated proteins of 48-hour liquid culture extracts in the presence of 30 mM $CaCl_2 \cdot 2H_20$ in the *in vitro* phosphorylation reactions with both the 'Hong' the 'Wang' labelling buffer (Plate III.3.9) were carried out. As was observed with the profiles obtained from phosphorylation in the presence of manganese, the labelling buffer did not appear to have a significant influence on labelling overall. When calcium was present in the labelling reaction, more efficient labelling was observed with the 'Hong' buffer but the 'Wang' labelling buffer appeared to be more efficient at labelling the protein with an apparent molecular weight of 68 kDa. The basis for the observed difference in labelling was not investigated further. The nitrocellulose membranes were stained with India ink to demonstrate the total protein profiles of these two-dimensional gels (Plate III.3.10).

Overall, the 'Hong' labelling buffer and temperature conditions resulted in more efficient labelling of phosphorylated proteins from *S. coelicolor* cell-free extracts. Due to problems with reproducibility, two-dimensional gel analysis was abandoned and alternative methods of characterizing the 32 kDa phosphorylated protein were investigated.

III.4 Western Analysis Using Anti-Phosphotyrosine Antibodies

SDS-polyacrylamide gel electrophoresis revealed significant differences between the phosphorylation status of a 32 kDa protein in the wild type (J1501) compared to the mutant (J1681). Therefore, the 32 kDa protein became the focus of research efforts. The phosphorylation state of this protein seems to be linked to development and since

Plate III.3.9. Characterization of proteins phosphorylated in the presence of $CaCl_2$ by two-dimensional gel electrophoresis. Proteins extracted from 48-hour liquid cultures of J1501 and J1681 were phosphorylated by [γ-^{32}P] in *in vitro* in reactions supplemented with 30 mM $CaCl_2$ in either 'Hong' labelling buffer or 'Wang' labelling buffer. (A) J1501 in 'Hong' labelling buffer (B) J1681 in 'Hong' labelling buffer (C) J1501 in 'Wang' labelling buffer (D) J1681 in 'Wang' labelling buffer. Proteins were subjected to 2-D gel analysis, transferred to nitrocellulose membranes, and autoradiographed for 2 days at -70°C with an intensifying screen. The pH range for isoelectric focusing (IEF) was pH 3 to pH 10 expanded in the pH 5 to pH 7 range. Molecular weights (in kDa) are shown.

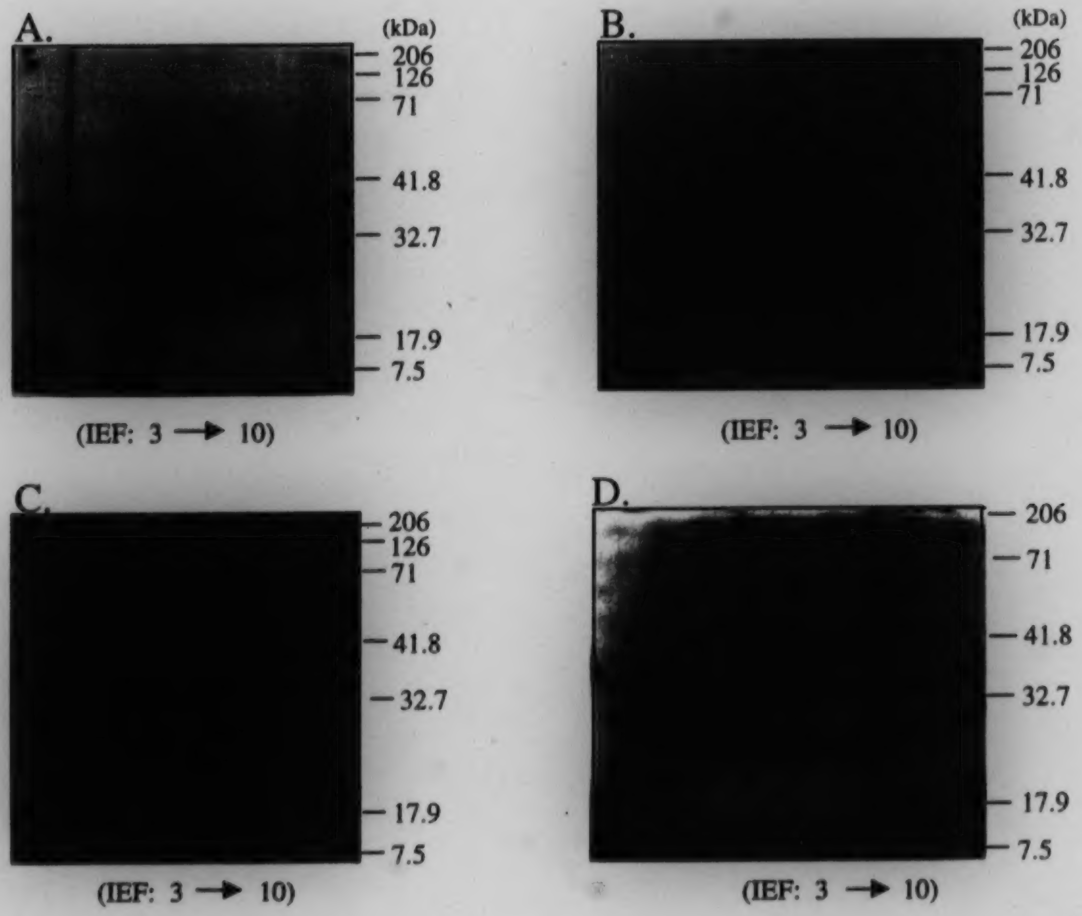
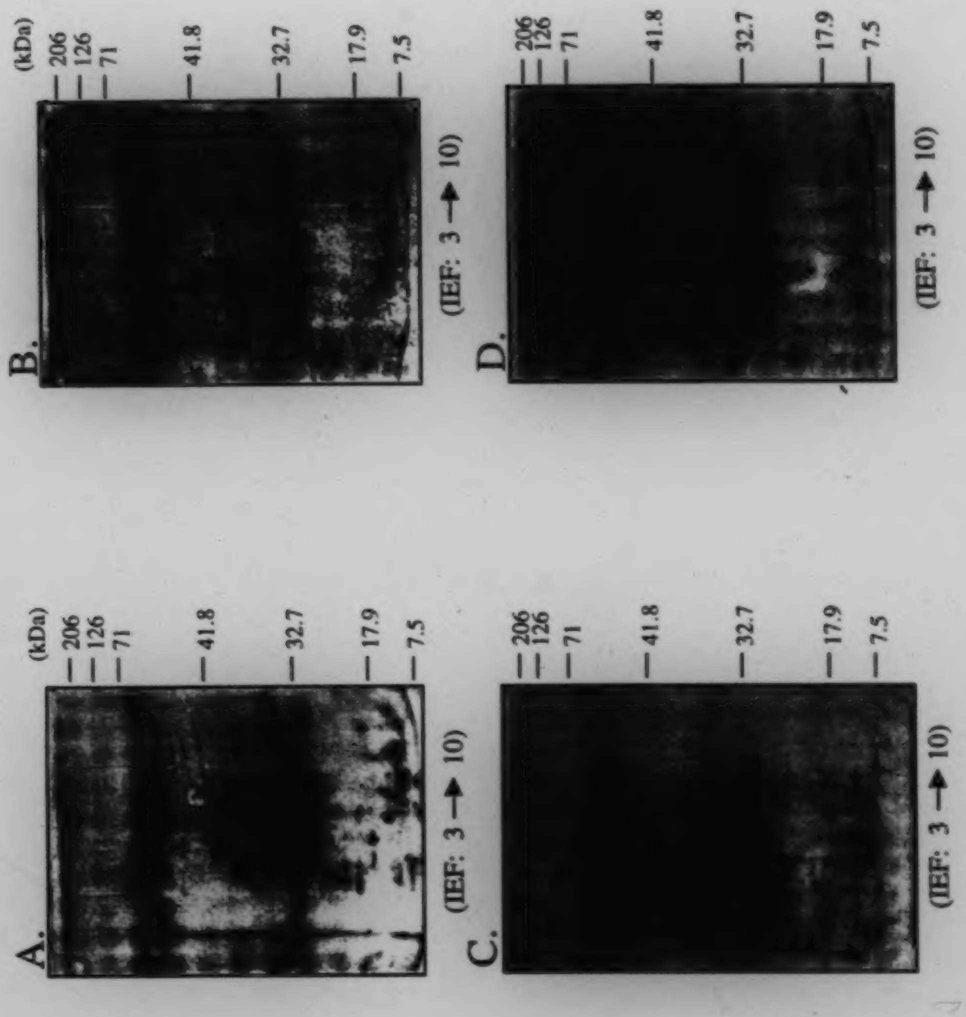

A.
(kDa)
206
126
71
41.8
32.7
17.9
7.5
(IEF: 3 → 10)
B.
(kDa)
206
126
71
41.8
32.7
17.9
7.5
(IEF: 3 → 10)
C.
206
126
71
41.8
32.7
17.9
7.5
(IEF: 3 → 10)
D.
206
71
41.8
32.7
17.9
7.5
(IEF: 3 → 10)

Plate III.3.10. India ink stained nitrocellulose membranes of two-dimensional gels of proteins phosphorylated in the presence of 30 mM $CaCl_2$. (A) J1501 in 'Hong' labelling buffer (B) J1681 in 'Hong' labelling buffer (C) J1501 in 'Wang' labelling buffer (D) J1681 in 'Wang' labelling buffer. Proteins were subjected to 2-D gel analysis, transferred to nitrocellulose membranes and stained with India ink. The pH range for isoelectric focusing (IEF) was pH 3 to pH 10 expanded in the pH 5 to pH 7 range. Molecular weights (kDa) are shown.



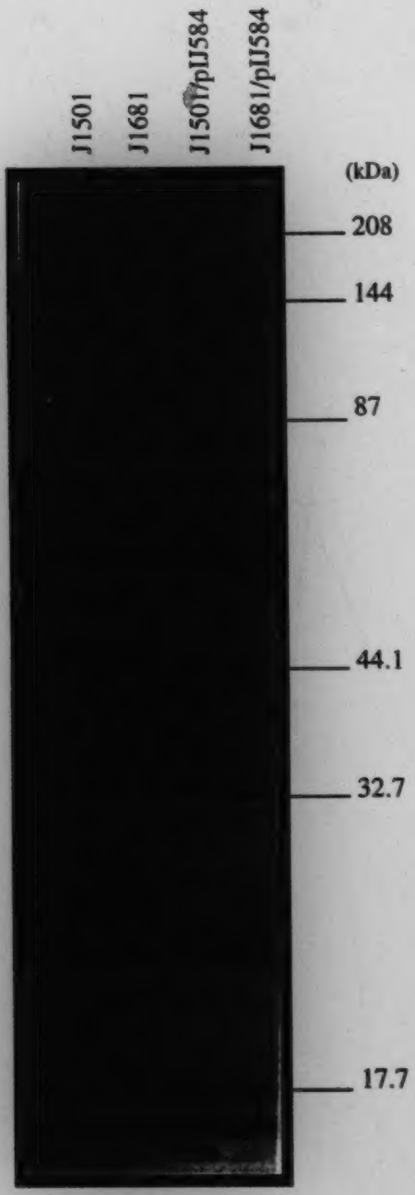
A.
B.
C.
D.
(kDa)
206
126
71
41.8
32.7
17.9
7.5
(IEF: 3 → 10)

tyrosine phosphorylation has been found to be associated with development in eukaryotes (Fantl *et al.*, 1993; Hunter and Cooper, 1985), it was of interest to determine if the 32 kDa protein displayed this type of phosphorylation.

III.4.1 Phosphotyrosine Profiles of Whole Cell-Free Extracts

In an attempt to characterize the 32 kDa protein, immunoblot analysis was performed using anti-phosphotyrosine antibodies (provided by Dr. H. Ostergaard). Proteins were separated on 10% SDS-polyacrylamide gels, transferred to PVDF membranes and probed with PY72, a monoclonal anti-phosphotyrosine antibody. Rabbit anti-mouse antibodies labelled with horseradish peroxidase were used as conjugate and visualization of phosphotyrosine on the Western blots was accomplished by the use of enhanced chemiluminescence. The 32 kDa protein was found to react with the anti-phosphotyrosine antibody, PY72 (Plate III.4.1.1). Interestingly, the amount of phosphotyrosine observed for the protein appeared to be equivalent for the mutant and the wild type unlike the profiles observed when the protein was labelled with [γ-^{32}P] ATP (Plate III.2.1.2, Plate III.2.1.3). The signal detected by enhanced chemiluminescence-based analysis has been demonstrated to be proportional to the amount of antigen present on the blot (Huang and Amero, 1997). The presence of a high copy number plasmid containing the *bldA* gene (pIJ584) did not influence the level of phosphotyrosine observed for the 32 kDa protein. Tyrosine, therefore, apparently does not account for the observed differences in phosphorylation between the *bldA*$^{+}$ and the *bldA*$^{-}$ strain indicating that in all likelihood, this was a multi- phosphorylated protein.

Plate III.4.1.1. Effect of *bldA* on tyrosine phosphorylation. Proteins from surface grown J1501 (*bldA*$^+$), J1681 (*bldA*$^-$), J1501/pIJ584, and J1681/pIJ584 were separated on a 10% SDS-polyacrylamide gel. Proteins were transferred to a PVDF membrane and probed with the anti-phosphotyrosine antibody preparation, PY72. Rabbit anti-mouse antibodies labelled with horseradish peroxidase were used as conjugate. Visualization of phosphotyrosine on the western blots was accomplished by the use of enhanced chemiluminescence (ECL). The high copy number plasmid, pIJ584 contains the *bldA* gene. Molecular weights (in kDa) are shown.


J1501
J1681
J1501/pIJ584
J1681/pIJ584
(kDa)
208
144
87
44.1
32.7
17.7

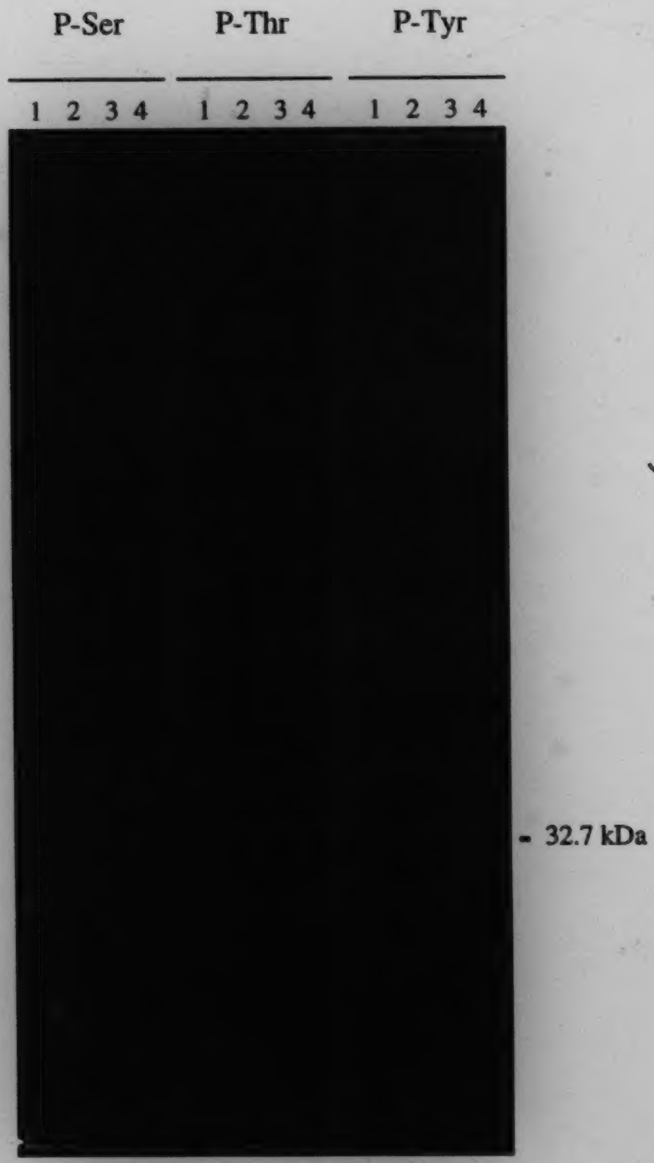
III.4.2 Blocking Experiments for the Confirmation of P-Tyrosine on the 32 kDa Protein

To confirm that the 32 kDa protein was indeed tyrosine phosphorylated and that the observed results were not due to non-specific binding of antibody, blocking experiments were performed in which the antibody was preincubated with free phosphoserine, free phosphothreonine and free phosphotyrosine. This experiment showed that free phosphotyrosine, but not free phosphoserine nor free phosphothreonine was able to block the binding of antibody to the 32 kDa protein (Plate III.4.2.1) indicating that this protein was very likely tyrosine phosphorylated.

III.5 Alkali-Enhanced Detection of Tyrosine and Threonine Phosphorylated Proteins

Western analysis had shown that the 32 kDa protein was likely phosphorylated on tyrosine and that the level of antibody bound to this protein was equivalent in both the wild type and the mutant strain. This was in contrast to the differences observed with *in vitro* labelling whereby the mutant appeared to be more highly phosphorylated. This seemed to indicate that the 32 kDa protein was phosphorylated on more than one amino acid residue. In order to gain insight into which residues may be involved in the phosphorylation of this protein, alkali enhancement experiments were performed. Phosphoserine is labile in alkali; therefore alkali enhancement can be used to enrich for phosphorylated tyrosine and threonine (Kamps, 1991) due to the stability to hydrolysis of these phosphoamino acids. To accomplish this, a PVDF membrane containing [γ-^{32}P] ATP labelled 32 kDa protein was submersed in 1M KOH and heated at 55°C for several

Plate III.4.2.1. Western analysis of surface culture proteins with phosphotyrosine antibodies after blocking with free phosphoamino acids. Proteins from 48-hour surface grown *S. coelicolor* (1) J1501, (2) J1681, (3) J1501/pIJ584, (4) J1681/pIJ584 were separated on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. The monoclonal PY72 antibody was preincubated with free phosphoserine (P-Ser), free phosphothreonine (P-Thr), and free phosphotyrosine (P-Tyr). Rabbit anti-mouse antibodies labelled with horseradish peroxidase were used as conjugate. Visualization of phosphotyrosine on the western blots was accomplished by the use of enhanced chemiluminescence (ECL). The high copy number plasmid, pIJ584 contains the *bldA* gene.


P-Ser
P-Thr
P-Tyr
1 2 3 4
1 2 3 4
1 2 3 4
32.7 kDa

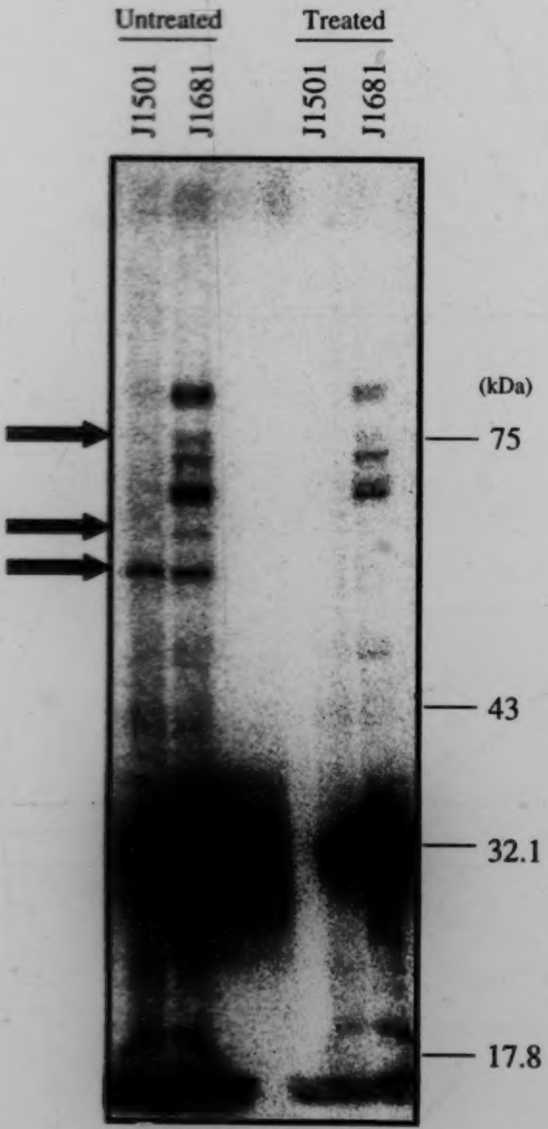
hours. After the blot was neutralized it was subjected to autoradiography. It was observed that the 32 kDa protein remained phosphorylated, even after alkali treatment (Plate III.5.1), indicating that it likely possesses phosphotyrosine and/or phosphothreonine. An equivalent protein load was used for this experiment as for the blocking experiment (compare Plate III.4.1.2 with Plate III.5.1) and supports the suggestion that a site other than tyrosine is responsible for the differences observed in the phosphorylation pattern between the wild type and the mutant. There are three phosphorylated protein bands that disappear upon treatment with 1 M KOH. It is likely that these proteins were phosphorylated on serine.

III.6 Phosphoamino Acid Analysis

Phosphotyrosine is not an abundant phosphoamino acid often making detection difficult (Hunter and Cooper, 1985). It had been established by Western analysis that the 32 kDa protein was likely phosphorylated on a tyrosine residue but the difference in total phosphorylation between the *bldA*$^+$ and the *bldA*$^-$ strain did not appear to be due to the phosphorylation of tyrosine. It was, therefore, important to determine specifically on what residues phosphorylation was occurring; especially since it had been reported that antibodies to phosphotyrosine can sometimes recognize phosphohistidine (Frackelton *et al.*, 1983).

Since the analysis by alkali enhancement and Western blotting indicated that the 32 kDa *bldA* target likely contained phosphorylated tyrosine and perhaps phosphorylated threonine residues, phosphoamino acid analysis was carried out to verify which amino

Plate III.5.1. Effect of alkali enhancement of protein phosphorylation profiles. *Streptomyces coelicolor* J1501 and J1681 whole cell free extracts from 48-hour surface grown cultures were electrophoresed on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. The last two lanes were treated in hot 1 M KOH. Both sections of the membrane were autoradiographed by overnight exposure to a phosphorimager screen. Grey arrows indicate phosphorylated bands that disappeared upon treatment with alkali. Molecular weights (in kDa) are shown on the right.


Untreated
Treated
J1501
J1681
J1501
J1681
(kDa)
75
43
32.1
17.8

acids were phosphorylated. During preliminary investigations with phosphoamino acid standards by thin layer electrophoresis it was determined that phosphothreonine and phosphotyrosine would be very difficult to resolve by one-dimensional thin layer electrophoresis (Plate III.6.1). Phosphoamino acid analysis, therefore, was performed using two-dimensional thin layer electrophoresis (Boyle *et al.*, 1991; Duclos *et al.*, 1991; Kamps, 1991). After separation of labelled proteins by sequential SDS-PAGE (see section III.8.3), the proteins were transferred to a PVDF membrane, the band corresponding to the 32 kDa protein was excised and then subjected to acid hydrolysis at 110°C. The acid hydrolysate was lyophilized, dissolved in non-radiolabelled phosphoamino acid standard solution containing P-serine, P-threonine, and P-tyrosine and finally, spotted onto thin layer cellulose plates. The samples were run in the first dimension with pH 1.9 buffer and in the second dimension with pH 3.5 buffer. The phosphoamino acid standards were visualized with ninhydrin and the plates exposed to a phosphorimager screen for 72 hours. The results of phosphoamino analysis (Plate III.6.2) indicated that phosphoserine, phosphothreonine and phosphotyrosine were all present on the 32 kDa-phosphorylated protein. This result was interesting for two reasons: first, phosphoserine was observed, yet with alkali treatment, the phosphorylation of the 32 kDa protein did not noticeably decrease, second, phosphotyrosine was observed but was very faint and four out of eight attempts at phosphoamino acid analysis resulted in no observable phosphotyrosine. This could be due to transient or unstable tyrosine phosphorylation of this protein, or it may have been the result of the small amount of protein that was loaded onto the thin layer chromatography (TLC) plates. The possibility exists that a co-purifying protein(s) may have contaminated the samples.

Plate III.6.1. Thin layer electrophoresis of phosphoamino acid standards. Aliquots of phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) alone and as a mixture, were spotted onto a cellulose thin layer chromatography sheet and electrophoresed in pH 1.9 electrophoresis buffer for 25 minutes at 1.5 kV. The sheet was dried, sprayed with ninhydrin and baked at 80°C.

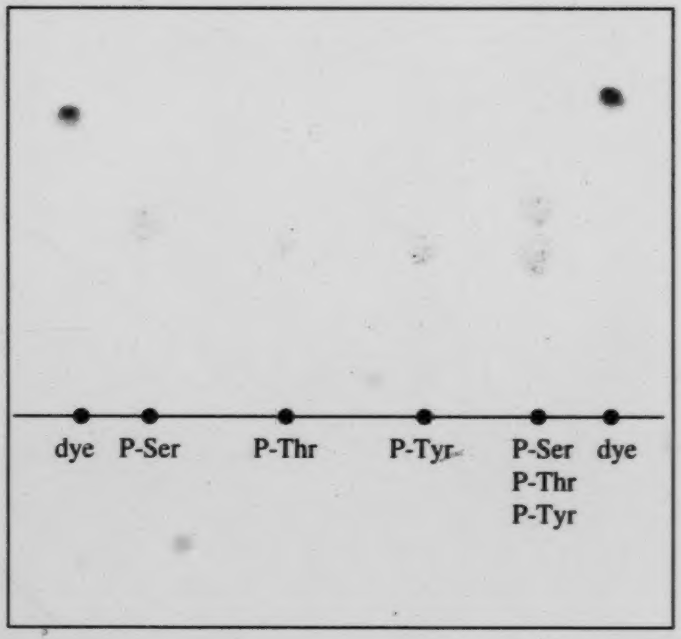
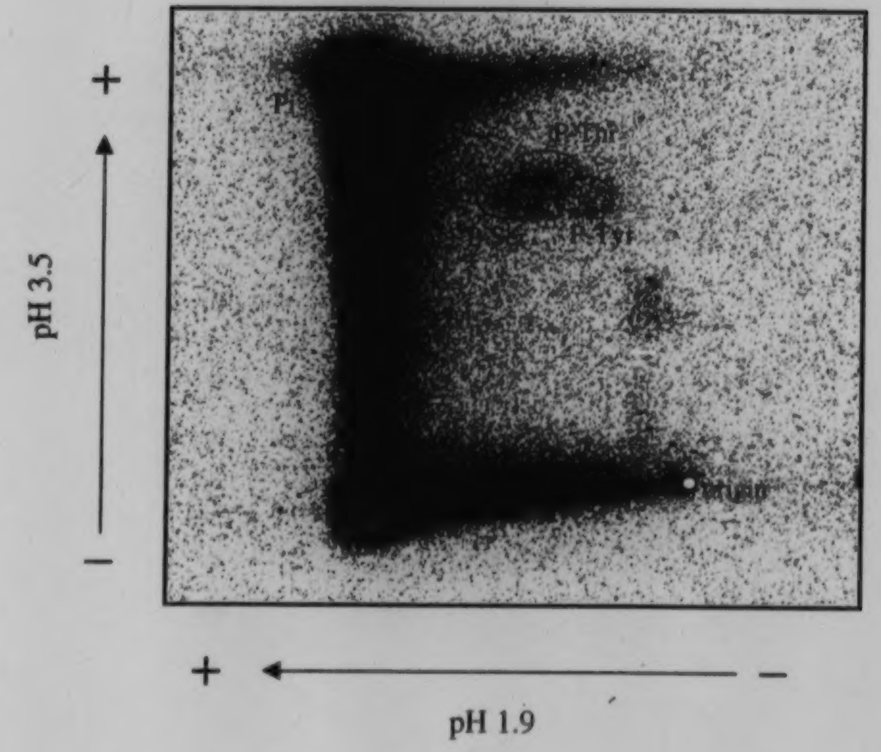

dye
P-Ser
P-Thr
P-Tyr
P-Ser
P-Thr
P-Tyr
dye

Plate III.6.2. Phosphoamino acid analysis of the 32 kDa protein by two-dimensional thin layer electrophoresis. PVDF membrane containing [γ-^{32}P] ATP labelled 32 kDa protein was hydrolyzed in 6N HCl at 110°C, lyophilized, dissolved in non-radiolabelled phosphoamino acid standard solution containing phosphoserine (P-Ser), phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr) and applied to thin layer cellulose plates. Samples were run in the first dimension with pH 1.9 buffer at 1.5 kV and in the second dimension with pH 3.5 buffer at 1.3 kV. The phosphoamino acid standards were visualized with ninhydrin by baking the TLC plate at 80°C. The radiolabelled phosphoamino acids were visualized by exposure to a phosphorimager screen for 72 hours. The positions of the unlabelled amino acids are shown by the circles and the origin is shown.


Pi
P-Thr
P-Tyr
Origin
+
pH 3.5
−
+
−
pH 1.9

III.7 Effect of *bldA* Complementation on Phosphorylation Profiles

It had been consistently observed that proteins extracted from *S. coelicolor* J1681 (*bldA*$^-$) became more phosphorylated in the *in vitro* phosphorylation experiments than did protein extracts from J1501 (*bldA*$^+$), therefore, the intensely phosphorylated protein at 32 kDa was of great interest. It appeared as though dephosphorylation of this protein was *bldA*-dependent since the protein was always phosphorylated to a greater extent in extracts from the *bldA* mutant strain, J1681. In order to determine whether dephosphorylation of this protein would occur with the introduction of the *bldA* gene into the *bldA*$^-$ strain, both J1501 and J1681 harboring a high copy number plasmid containing the *bldA* gene (Leskiw *et al.*, 1993), pIJ584, were grown as surface cultures and cell-free extracts were prepared. When pIJ584 was transformed into J1681, the strain was no longer bald and exhibited a wild type phenotype with sporulation and pigment production that was comparable to the wild type, J1501. The presence of *bldA* at high copy in J1501, which was wild type for *bldA*, did not exhibit any phenotypic differences from untransformed J1501. The phosphorylation profiles of the transformed strains were compared with those of the plasmid-free J1501 and J1681 strains. In order to verify that the *bldA* gene and not the plasmid vector was responsible for the restored sporulation and any differences in the phosphorylation profile, the plasmid pIJ486 without insert was also transformed into J1501 and J1681. As expected, the vector without the insert containing the *bldA* gene did not complement the mutant phenotype nor did it significantly alter the phosphorylation profile. If the phosphorylation state of the 32 kDa protein was *bldA*-dependent then it would be expected that an increase in *bldA* copy number would result in a decrease in the amount of phosphorylation observed for the 32 kDa protein in J1681 to the levels observed with the wild type strain (Plate III.7.1). A decrease was observed in the intensity of phosphorylation for the 32 kDa protein when *bldA* was present in high copy (Plate III.7.1). These results suggest that J1681 lacks a *bldA*-dependent phosphatase. What was not expected was that the wild type strain, when transformed with pIJ584, exhibited an increase in the amount of phosphorylation of the 32 kDa protein to a level observed with the *bldA*-deleted mutant. It is possible that the increase in the phosphorylation state of the 32 kDa protein that resulted when the *bldA* gene was present at high copy in the wild type strain, J1501 was a result of titrating out the *bldA*-dependent phosphatase. This would seem to indicate that the regulation of the phosphorylation state of this protein occurs at more than one level.

III.8 Purification of the Potential 32 kDa *bldA* Target

The 32 kDa-phosphorylated protein was chosen for characterization because there was a very significant difference between the phosphorylation state of this protein between the wild type, J1501, and the mutant, J1681. Because the goal was to be able to clone and characterize the gene for this protein, peptide sequence was required so that oligonucleotide probes could be designed. To accomplish this, pure protein was required. Three procedures were attempted for the purification of this protein: chromatography, electroelution, and a sequential band excision/electrophoresis method.

Plate III.7.1. Effect of *bldA* on phosphorylation of the 32 kDa protein. Whole cell-free extracts of 48-hour surface grown cultures of J1501/pIJ486, J1681/pIJ486, J1501 (*bldA*$^+$), J1501/pIJ584, J1681 (*bldA*$^-$), and J1681/pIJ584 were labelled with [γ-^{32}P] ATP, separated on 10% SDS-polyacrylamide gels, transferred to PVDF membranes and exposed overnight to film at –70°C with an intensifying screen. The high copy number plasmid, pIJ584 contains the *bldA* gene.

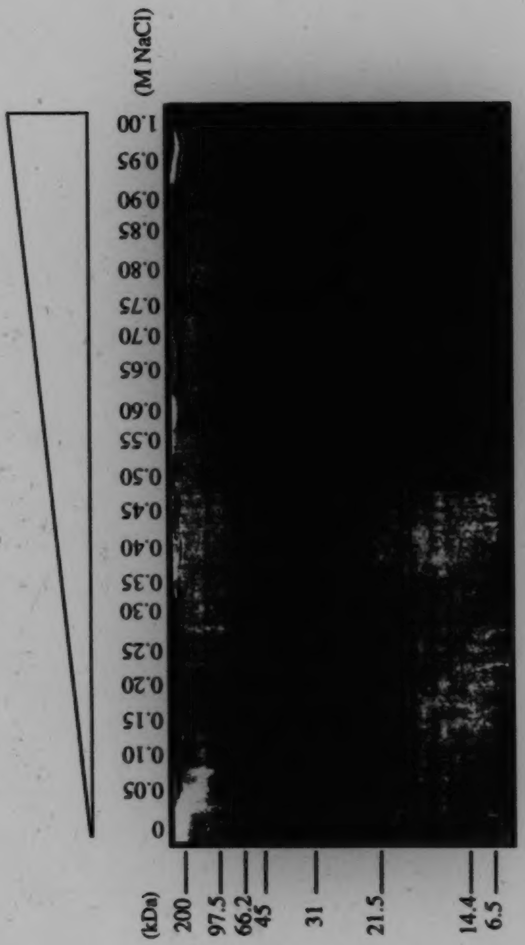
J1501/pIJ486
J1681/pIJ486
J1501
J1501/pIJ584
J1681
J1681/pIJ584
(kDa)
71
32.1

III.8.1 Chromatographic Separation

Anion-exchange chromatography was chosen to accomplish purification of the 32 kDa protein. Because the protein of interest was phosphorylated, column chromatography had to be carried out with [γ-^{32}P] ATP-labelled protein. In order to make this task easier, preliminary work was performed with unlabelled protein from whole cell-free extracts of 48-hour surface cultures so that chromatographic conditions for the separation of radiolabelled proteins could be determined. An NaCl gradient was used for elution of proteins from the column. It was therefore a concern that acetone precipitation may concentrate the salt and adversely influence the electrophoretic mobility of precipitated proteins. To determine if this would be a problem, the effect of NaCl concentration on acetone precipitation and subsequent electrophoresis of proteins was investigated. Various concentrations of NaCl ranging from 0 to 1.00 M (final concentration) were added to 10 µg aliquots of protein from 48-hour J1501 extracts, and subjected to acetone precipitation. Acetone was evaporated from the precipitates and the pellets were redissolved in 25 µL 10 mM HEPES and 5 µL aliquots were loaded onto a 12% SDS-polyacrylamide gel. Higher molecular weight proteins did not appear to be precipitated as efficiently with higher NaCl concentrations (Plate III.8.1.1) but electrophoretic mobility was not affected.

Some acetone precipitation protocols (Bollag and Edelstein, 1993) report that 5 volumes of acetone at –20°C could quantitatively precipitate less than 1 µg of protein. Since the amount of 32 kDa phosphorylated protein was expected to comprise only a small proportion of total CFEs, it was of interest to investigate this possibility. Increasing

Plate III.8.1.1. Effect of sodium chloride concentration on acetone precipitation of proteins. Aliquots containing 10 μg protein from surface culture cell-free extracts of *S. coelicolor* J1501 containing increasing concentrations of NaCl were precipitated with 2 volumes of acetone and subjected to electrophoresis on a 12% SDS-polyacrylamide gel and stained with Coomassie blue. Molecular weights (in kDa) are shown.


(M NaCl)
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
(kDa)
200
97.5
66.2
45
31
21.5
14.4
6.5

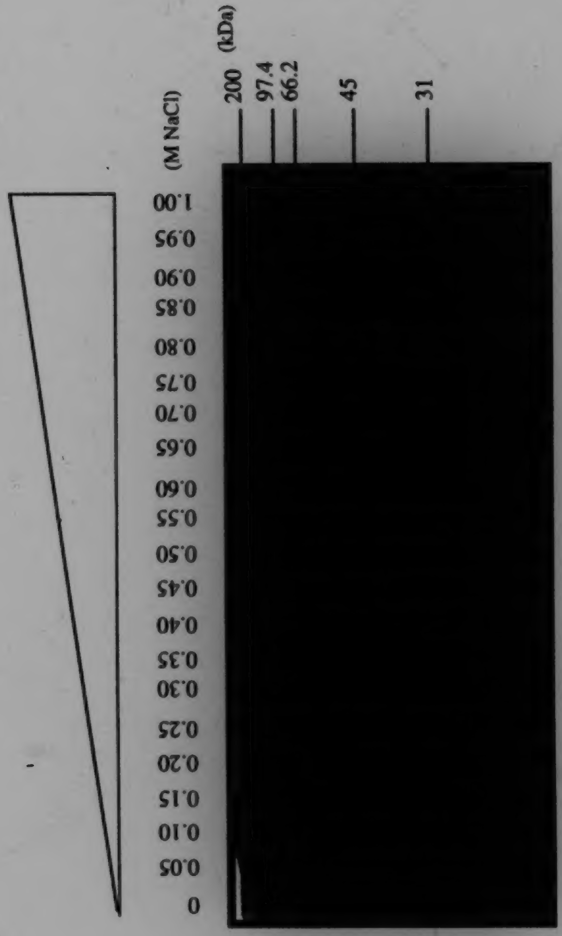
the amount of acetone used to precipitate the proteins in the presence of NaCl from 2 volumes to 5 volumes appeared not to be advantageous (Plate III.8.1.2) since at NaCl concentrations greater than 0.80 mM, several proteins were not recoverable. Sodium chloride concentration, therefore, did indeed exhibit an effect on recovery of proteins by acetone precipitation. The volume of acetone used for protein precipitation did not appear to adversely influence recovery of proteins in the presence of low NaCl concentrations (less than 0.35 mM).

In order to determine the conditions required for the chromatographic separation of proteins from whole CFEs of 48-hour surface cultures, unlabelled CFE was used. An aliquot containing 0.5 mg of total protein was applied to DEAE Sepharose FF columns and proteins were eluted with a linear NaCl gradient. Figure III.8.1.3 shows a typical elution profile for the anion-exchange chromatography of unlabelled J1501 48-hour surface culture CFEs. The peaks of protein elution were reproducible with respect to the NaCl concentration at which they eluted.

Twelve major protein peaks were observed when fractions were analyzed spectrophotometrically at A_{280}. Two-dimensional gels of pooled fractions (Plate III.8.1.4) revealed that six of the 12 pooled fractions contained a 32 kDa protein (fractions V, VI, VII, VIII, IX, X, XI), indicating that adequate separation of proteins in the 32 kDa range was not achieved. It was therefore important that radioactively labelled protein be subjected to chromatography to determine which fraction(s) contained the 32 kDa protein of interest.

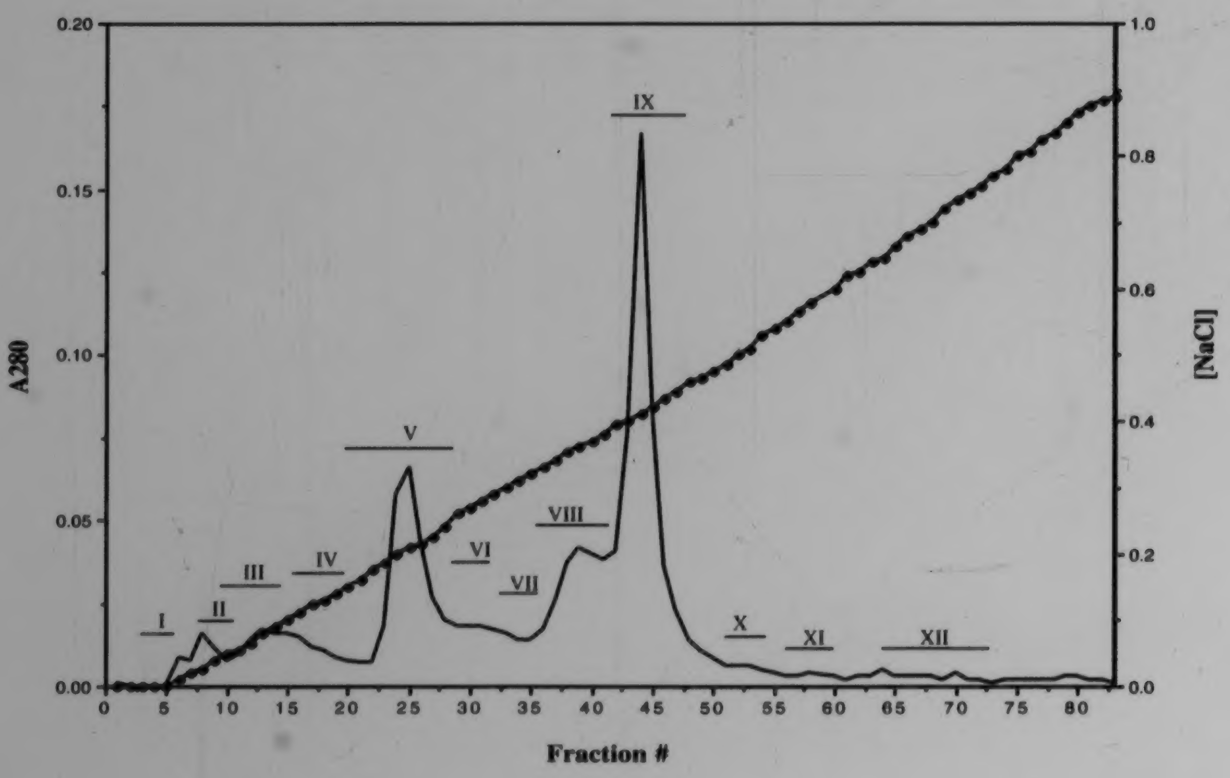
Since the protein of interest was phosphorylated, radiolabelled CFEs were used for chromatography employing the same elution conditions as those used for the

Plate III.8.1.2. Effect of acetone volume on the precipitation of proteins in the presence of sodium chloride. Aliquots containing 10 µg protein from surface culture cell-free extracts of *S. coelicolor* J1501 containing increasing concentrations of NaCl were precipitated with 5 volumes acetone and subjected to electrophoresis on a 12% SDS-polyacrylamide gel and stained with Coomassie blue. Molecular weights (in kDa) are shown.



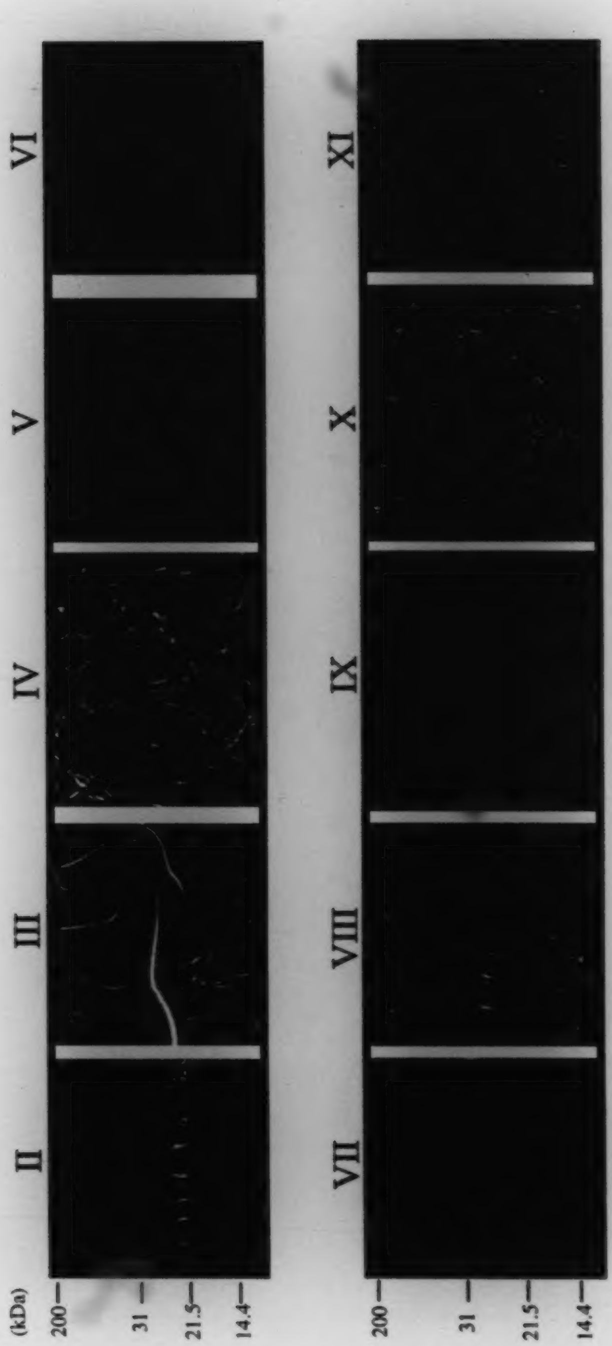
(M NaCl)
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
(kDa)
200
97.4
66.2
45
31

Figure III.8.1.3. Elution profile of proteins extracted from *Streptomyces coelicolor* J1501 48-hour surface cultures by anion exchange chromatography. An aliquot of whole cell-free extract containing 0.5 mg protein was applied to a DEAE-Sepharose FF column and eluted with a linear NaCl gradient. Fractions of the eluate were collected and analyzed for protein content (——) and NaCl concentration (◆). Roman numerals refer to pooled fractions.



0.20
0.15
0.10
0.05
0.00
A280
1.0
0.8
0.6
0.4
0.2
0.0
[NaCl]
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
Fraction #
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII

Plate III.8.1.4. Two-dimensional gel analysis of protein fractions eluted by anion-exchange chromatography. Roman numerals correspond to pooled fractions eluted from a DEAE-Sepharose FF column (see Figure III.8.1.3). No detectable protein was observed in fractions I and XII and are not shown. Gels were silver stained and preserved in BioGel wrap. Molecular weights (kDa) are shown. The pH range for isoelectric focusing (IEF) was pH 3 to pH 10 expanded in the pH 5 to pH 7 range.


II
III
IV
V
VI
VII
VIII
IX
X
XI
(kDa)
200
31
21.5
14.4
200
31
21.5
14.4

unlabelled extracts. The pattern of elution of radioactive fractions from the DEAE-Sepharose FF column is shown in Figure III.8.1.5. There were five major peaks of radioactivity eluted. When SDS-polyacrylamide gel analysis of radioactive fractions was performed and proteins were transferred to a PVDF membrane, stained with India ink and autoradiographed, no phosphorylated proteins were observed. India ink staining of the membrane revealed protein bands, but none were in the 32 kDa range (Plate III.8.1.6). The fractions with the highest counts, as determined by LSC, eluted from the column within the first fifteen fractions yet there were no detectable phosphorylated proteins. This seems to indicate that label had been released from phosphorylated proteins prior to SDS-PAGE (labelled reactions were dialyzed to get rid of unincorporated label). Replicate columns gave a similar elution pattern of radioactivity from the column as the one exhibited in Figure III.8.1.5. It could be that fractions containing peaks of the radioactively labelled proteins that eluted from the column contained low molecular weight labelled proteins and that they ran off the gel. It was also a possibility that phosphorylation was not stable under the chromatography conditions used. The label may have been released from the proteins at the start of chromatographic separation and the first fractions eluted contained unincorporated label that had been released from the proteins. Spectrophotometric quantification of protein levels in the radioactive fractions was not attempted. It was also a possibility that other radioactive proteins were irreversibly bound to the column bed since residual radioactivity was difficult to remove from the column (Figure III.8.1.5). Quantification of protein levels in each of the pooled fractions revealed that twenty-four percent of the protein was unaccounted for in cold

Figure III.8.1.5. Elution of [γ-^{32}P] ATP-labelled protein fractions from a DEAE-Sepharose FF column. Cell-free extracts from 48-hour surface cultures of *S. coelicolor* J1501 were labelled *in vitro* by [γ-^{32}P] ATP and subjected to anion-exchange chromatography. Proteins were eluted with a linear NaCl gradient. The amount of radioactivity in fractions was determined by liquid scintillation counting. Roman numerals correspond to protein peaks determined for chromatography of unlabelled proteins of identical extracts (see Figure III.8.1.3).

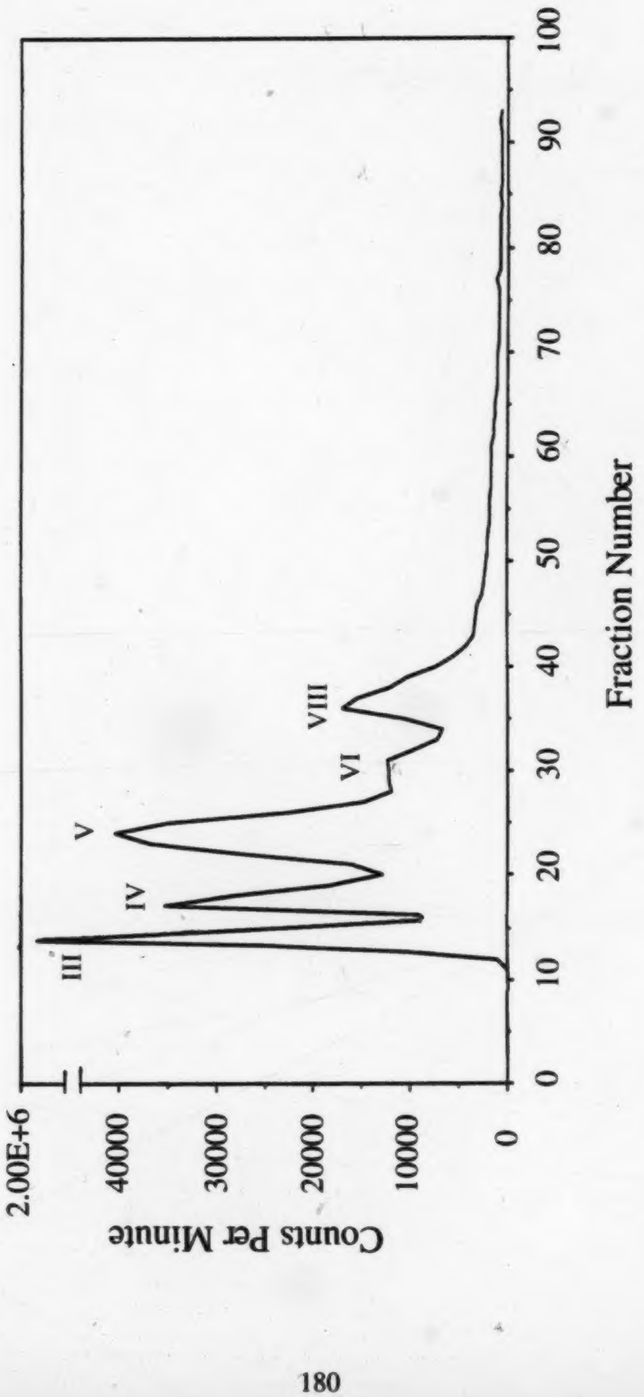
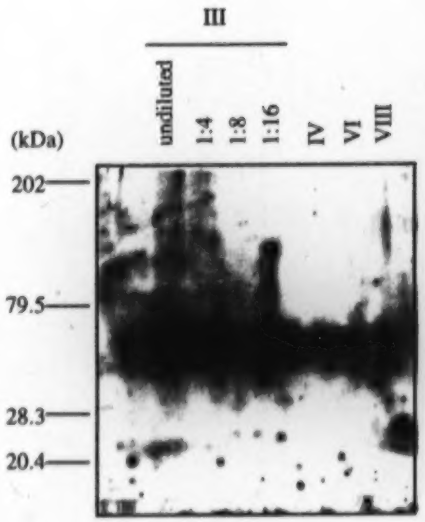
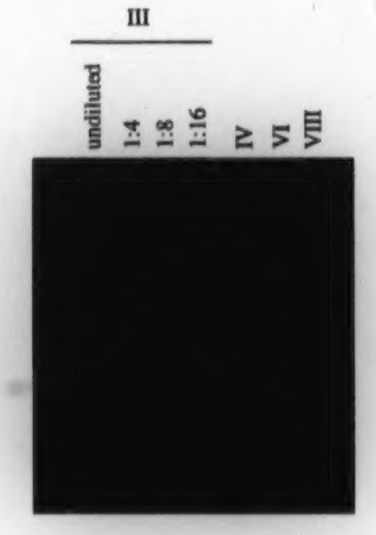

Counts Per Minute
2.00E+6
40000
30000
20000
10000
0
Fraction Number
0
10
20
30
40
50
60
70
80
90
100
III
IV
V
VI
VIII

Plate III.8.1.6. Analysis of labelled fractions eluted from a DEAE-Sepharose FF column by SDS-polyacrylamide gel electrophoresis. Aliquots corresponding to pooled fractions containing radioactivity which eluted during anion-exchange column chromatography were separated on 10% SDS-polyacrylamide gels, transferred to a PVDF membrane and autoradioagraphed. (A) The Western blot was stained with India ink. Fraction pool III, which contained almost two million counts, was analyzed undiluted and diluted. (Roman numerals correspond to protein peaks determined for chromatography of unlabelled proteins of identical extracts (see Figure III.8.1.3). (B) Autoradiogram of the Western blot.


III
undiluted
1:4
1:8
1:16
IV
VI
VIII
(kDa)
202
79.5
28.3
20.4

A.


III
undiluted
1:4
1:8
1:16
IV
VI
VIII

B.

(unlabelled protein) experiments so it was possible that this percentage represented phosphorylated proteins which had adhered irreversibly to the column.

Chromatography was not a successful method for the purification of the 32 kDa-phosphorylated protein. Proteins were irreversibly bound to the column, therefore all the proteins present in the control reaction *versus* the proteins that eluted from the column could not be accounted for. Phosphorylated proteins were not detected by SDS-PAGE. After several unsuccessful attempts, this method was abandoned in favor of electroelution (III.8.2).

III.8.2 Electroelution

Since chromatography did not prove to be a satisfactory method by which to purify the 32 kDa phosphorylated protein, an alternate method, the electroelution of the protein from an SDS-polyacrylamide gel slice, was performed. Radioactive samples could not be sent for N-terminal amino acid sequencing, therefore, labelled reactions were analyzed alongside the unlabelled proteins for use as size comparisons so that the correct band would be excised from the gel.

Eight lanes, each containing 100 µg of protein, and one lane of *in vitro* labelled protein of 48-hour surface culture cell-free extracts were electrophoresed on a 10% SDS-PAG, and stained with Coomassie blue stain. The stained gel was exposed overnight to X-ray film. The autoradiogram was aligned with the gel such that the phosphorylated band corresponding to the 32 kDa protein could be used as a reference so that the unlabelled protein could be excised. Great care was taken to excise a very narrow gel slice of the appropriate size. The excised protein was then subjected to electrophoretic elution at 4°C and then to dialysis. The protein was removed from the sample collection well and two aliquots equivalent to 10% of the sample were removed. The samples were evaporated to dryness in a SpeedVac, and washed three times in Milli-Q water. The pellet corresponding to the 10% aliquot was dissolved in 3X Laemmli's Final Sample buffer and loaded onto a 10% SDS-polyacrylamide gel. This method did yield a protein band of the correct size (Plate III.8.2.1-A). Two-dimensional analysis was not performed on the purified protein. Western analysis of the purified protein was carried out. A PVDF membrane containing the 32 kDa purified protein was probed with the anti-phosphotyrosine antibody, PY72, with rabbit anti-mouse antibodies labelled with horseradish peroxidase used as conjugate. Visualization of phosphotyrosine was accomplished by enhanced chemiluminescence. The purified protein reacted with the anti-phosphotyrosine antibodies and was therefore phosphorylated on tyrosine (Plate III.8.2.1-B). Due to the small amount of protein recovered by this method and due to the amount of protein required for amino acid sequencing, no attempt was made to assay phosphorylation of the pure protein.

The purified 32 kDa protein was sent to Ms. S Kielland, Department of Biochemistry and-Microbiology, University of Victoria, Victoria, British Columbia for N-terminal sequencing. Several unsuccessful attempts were made at sequencing this protein because it was found to be N-terminally blocked.

Because more than one protein band was recovered by this procedure (compare Plate III.8.2.1-A with -B), an alternate method of purification was investigated.

Plate III.8.2.1. Electrophoretic elution of the 32 kDa protein from an SDS-polyacrylamide gel. (A) An aliquot of purified 32 kDa protein obtained from electroelution analyzed by electrophoresis on a 10% SDS-PAG and silver stained. (B) Western analysis of the 32 kDa protein purified by electroelution. PY72 anti-phosphotyrosine antibodies were used to probe a PVDF membrane containing the purified protein. Rabbit anti-mouse antibodies labelled with horseradish peroxidase were used as conjugate. Visualization of phosphotyrosine on the western blot was accomplished by the use of chemiluminescence.

B.
32 kDa
A.

III.8.3 Sequential-Gel Band Excision Purification of the 32 kDa Protein

Because column chromatography proved to be problematic for the purification of the phosphorylated 32 kDa protein, and because it was difficult to resolve a single 32 kDa protein on a one-dimensional gel, a sequential-gel band purification strategy (as suggested by Dr. K.L. Roy, personal communication) was employed. This method utilized one-dimensional gels of different percentages in an attempt to purify the 32 kDa-phosphorylated protein. Sodium thioglycollate at 0.1 mM was added to the top electrophoresis reservoir buffer to scavenge any free radicals that may be present in the gel, which may contribute to N-terminal blockage. A lane of *in vitro* phosphorylated protein was run alongside unlabelled protein samples of 48-hour surface culture cell-free extracts on a 10% SDS-PAG for comparison to ensure the correct protein was purified. Following exposure to a phosphorimager screen, the autoradiogram and the gel were aligned so that the protein corresponding to the 32 kDa phosphorylated protein could be excised. A 15% SDS-PAG was poured around the excised gel slices that had been positioned between two glass plates. This was found to be more effective than trying to position the gel slices onto a previously poured gel. Once electrophoresis on the 15% SDS-PAG was complete, the gel was transferred to a PVDF membrane, stained with Coomassie brilliant blue and exposed to a phosphorimager screen. Two Coomassie brilliant blue-stained protein bands were observed. The protein band corresponding to the radiolabelled 32 kDa band of interest was excised from the PVDF membrane, rinsed with Milli-Q water and sent to Ms. S Kielland, Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia for N-terminal sequencing. This protein was again found to be N-terminally blocked. An overall amino acid composition analysis was performed by the facility and revealed that the 32 kDa protein consisted of 308 amino acids with an estimated molecular weight of 32,181 Daltons. This suggested that a single protein was present since if a contaminating protein was present in the sample it would be expected that the total amino acid analysis would indicate a larger molecular weight than expected.

Although the N-terminal sequence could not be determined for this protein, it was suggested by Dr. G. Wright (personal communication) that the protein be sent for internal peptide sequencing at the Harvard Microchemistry Facility. After consultation with Dr. William Lane of this facility, the 32 kDa band was purified by sequential gel electrophoresis as described above but the gels were subjected to 10 minutes of staining with Coomassie blue. The very centre portion of the stained protein band corresponding to the radiolabelled 32 kDa protein was excised. Gel slices of an equivalent area, representing background were removed directly beneath the position of the 32 kDa band to serve as a control for co-purifying, contaminating proteins. The excised gel slices were washed twice in 50% acetonitrile, flash frozen and an estimated 300 pmol was sent for microsequencing.

Two internal peptide sequences were obtained: TMGHAGAIVSGSSGTAQAK and ATVGEVGNAEQSNINWGK. A database search did not reveal any homology to known proteins at the time these peptide sequences were obtained.

III.9 Attempted Isolation of the Gene for the 32 kDa Protein Using Oligonucleotide Probes

Efforts to clone the gene for the 32 kDa protein involved the investigation of several cloning and screening approaches.

III.9.1 Preparation and Hybridization of the Oligonucleotide Probes to Genomic Digests of *S. coelicolor* –Trial #1

Oligonucleotide probes based on the two internal amino acid sequences obtained from sequencing the 32 kDa protein were designed based on the codon usage table for *Streptomyces* (Wright and Bibb, 1992) and synthesized by the Department of Biological Sciences Synthesis Service, University of Alberta. JLS2, an oligonucleotide guessmer based on the amino acid sequence MGHAGAIV, with the sequence 5'-ATGGG(CG)CACGC(CG)GG(CG)GC(GC)ATCGT-3' and JLS3, corresponding to GEVGNAEQ with the sequence 5'-GG(CG)GAGGT(CG)GG(GC)AACGC(CG)GAGCAG-3' were end-labelled and used as probe to screen chromosomal digests of wild type *S. coelicolor* DNA by Southern hybridization (Plate III.9.1.1 and Plate III.9.1.2). Comparison of the membranes hybridized with JLS2 (Plate III.9.1.1-A, Plate III.9.1.2-A) and JLS3 (Plate III.9.1.1-B, Plate III.9.1.2-B) revealed a common hybridizing 1.7 kb *Sst*II fragment. This fragment was chosen for initial cloning experiments.

III.9.2 Cloning of the Hybridizing Fragments – Trial #1

A fragment of approximately 1.7 kb was found to hybridize to both of the JLS2 and JLS3 oligonucleotide probes (Plate III.9.1.1 and Plate III.9.1.2). Chromosomal *Sst*II-

Plate III.9.1.1. Autoradiograms of the Southern blots of *Streptomyces coelicolor* J1501 genomic DNA after hybridization with ^{32}P-labelled oligonucleotide probes. Eleven identical aliquots of *S. coelicolor* J1501 genomic DNA were digested with a variety of restriction endonucleases and then subjected to electrophoresis on a 1% agarose gel. The DNA fragments were then transferred to a Hybond-N nylon membrane and probed separately with ^{32}P-labelled JLS2 (A) at 50°C in 50% formamide hybridization buffer and JLS3 (B) at 50°C in 40% formamide hybridization buffer. The membranes were washed twice for 30 minutes in 2X SSC. 0.1% SDS solution and twice for 30 minutes in 0.2X SSC, 0.1% SDS solution. Size markers are shown in kb.

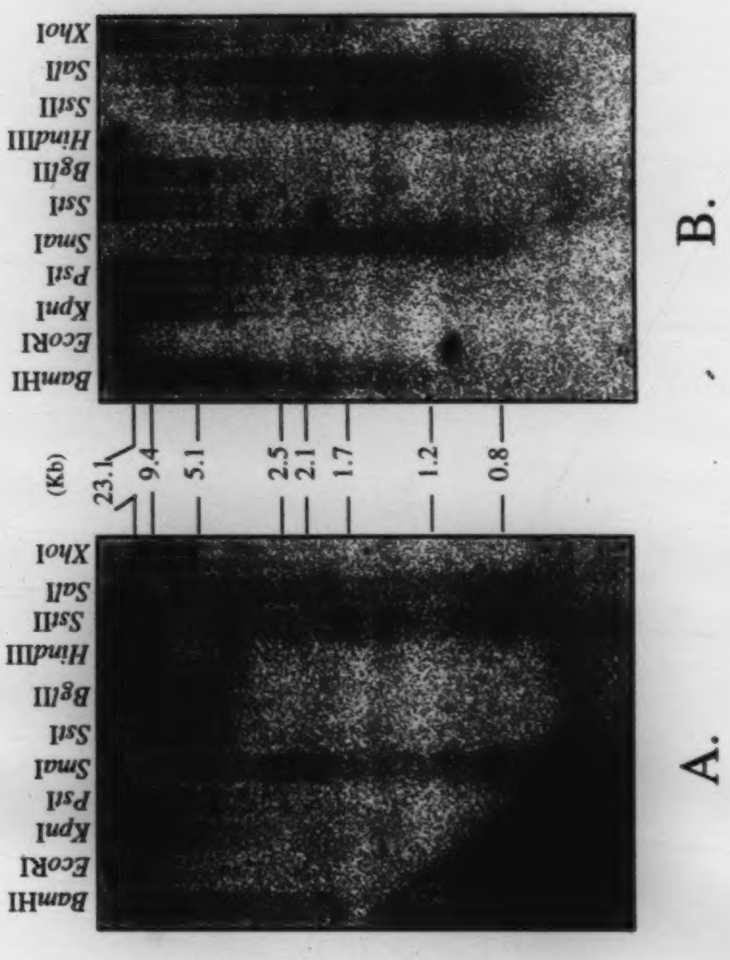
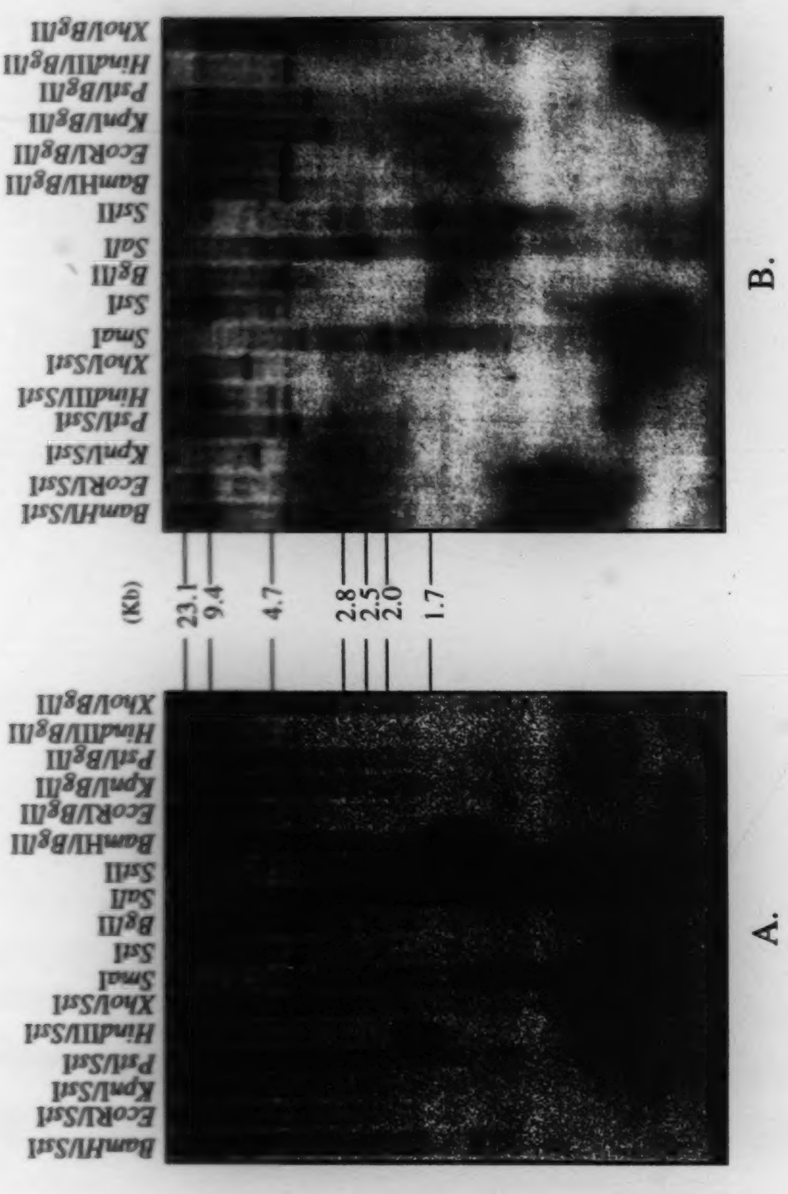

A.
B.
BamHI
EcoRI
KpnI
PstI
SmaI
SstI
BglII
HindIII
SstII
SalI
XhoI
(Kb)
23.1
9.4
5.1
2.5
2.1
1.7
1.2
0.8

Plate III.9.1.2. Autoradiograms of *Streptomyces coelicolor* J1501 genomic DNA after hybridization with ^{32}P-labelled oligonucleotide probes. Seventeen identical aliquots of *S. coelicolor* J1501 genomic DNA were digested with a variety of restriction endonucleases, singly and in pairs, and then subjected to electrophoresis on a 1% agarose gel. The DNA fragments were then transferred to a Hybond-N nylon membrane and probed separately with ^{32}P-labelled JLS2 (A) at 50°C in 50% formamide hybridization buffer and JLS3 (B) at 50°C in 40% formamide hybridization buffer. The membranes were washed twice for 30 minutes in 2X SSC. 0.1% SDS solution and twice for 30 minutes in 0.2X SSC, 0.1% SDS solution. Size markers are shown in kb.


A.
B.
BamHI/SstI
EcoRI/SstI
KpnI/SstI
PstI/SstI
HindIII/SstI
XhoI/SstI
SmaI
SstI
BglII
SalI
SstII
BamHI/BglII
EcoRI/BglII
KpnI/BglII
PstI/BglII
HindIII/BglII
XhoI/BglII
(Kb)
23.1
9.4
4.7
2.8
2.5
2.0
1.7

digested fragments of this size range were purified by the crush and soak method (Sambrook *et al.*, 1989), and shotgun cloned into similarly digested pBluescript KS$^+$ and transformed into *Escherichia coli* DH5α. Transformants were screened by colony hybridization using the two oligonucleotides (JLS2 and JLS3) as probes and one strongly hybridizing clone was identified from the 900 colonies screened. That this clone (A4-66) contained a ~1.7 kb *Sst*II fragment was verified by digestion of the isolated plasmid DNA with *Sst*II. To confirm that tandem ligation of *SstII* fragments into the vector had not occurred, the plasmid DNA was also digested with *Xba*I and *Sac*I, restriction enzymes with sites flanking the *Sst*II site, to reveal a ~1.7 kb insert. Double-stranded DNA of this clone was sent for sequence analysis (Department of Biological Sciences Sequencing Service, University of Alberta).

Attempted sequence analysis of pA4-66 with JLS2 and JLS3 as sequencing primers was not successful. Furthermore, sequence analysis of this plasmid DNA with both forward and reverse universal primers did not reveal any sequences matching the oligonucleotide sequences of the probes. A database search of the nucleotide sequence against the Sanger Centre database placed it on cosmid SC17 (Table II.2.1.3). The corresponding protein sequence on the cosmid was SC17.02, a possible oxidoreductase of 281 amino acids. Since the hybridization conditions led to the isolation of a false-positive clone, the stringency conditions for hybridization were obviously not sufficiently high. Due to this unsatisfactory result, it was decided that the primers would be redesigned.

III.9.3 Preparation and Hybridization of the Oligonucleotide Probes to Genomic Digests of *S. coelicolor* –Trial#2

Since the A4-66 clone represented the only potential positive clone out of 900 screened but did not yield a clone with sequence corresponding to the probes, two 50-mer oligonucleotide probes were designed based on the peptide sequence ATVGEVGNAEQSNINWGK and were essentially longer versions of JLS3. The redesigned 50-mer oligonucleotide probes were: JLS16 (5'-GC(GC)AC(GC)GT(GC)GG(GC)GAGGT(GC)GG(GC)AACGC(GC)GAGCAGAGCAACATCAACTACGG-3') with 128-fold degeneracy and JLS17 (5'-GC(GC)AC(GC)GT(GC)GG(GC)GAGGT(GC)GG(GC)AACGC(GC)GAGCAGTC(GC)AACATCAACTACGG-3') with 256-fold degeneracy.

As before for JLS2 and JLS3, these two oligonucleotide probes were used to probe *S. coelicolor* J1501 chromosomal digests. Because of the disappointing results of the initial attempt at cloning the gene encoding the 32 kDa protein, conditions of varying stringency were tested for the two 50-mer oligonucleotide probes (JLS16 and JLS17) in an attempt to avoid falsely positive hybridizing fragments. Four identical Southerns of *S. coelicolor* J1501 chromosomal DNA that had been digested with restriction endonucleases and transferred to Hybond-N membranes were probed with [γ-^{32}P] ATP end-labelled JLS16 (Plate III.9.3.1) and JLS17 (Plate III.9.3.2) at 45°C in hybridization solution containing 20%, 30%, 40%, and 50% formamide. The membranes were washed twice for 30 minutes in 2X SSC, 0.1% SDS and twice for 30 minutes in 0.2X SSC, 0.1% SDS. Under these conditions, it appeared as though for the JLS16 probe, 50% formamide in the hybridization buffer was best while for the JLS17 probe, 40% formamide in the hybridization buffer appeared to yield the best hybridization profile.

Plate III.9.3.1. Autoradiograms of Southern blots of restriction enzyme digested *S. coelicolor* J1501 genomic DNA probed with JLS16 in the presence of formamide. Four sets of seven identical aliquots of *S. coelicolor* DNA digested with *Sac*I, *Sac*II, *Sal*I, *Sma*I, *Pst*I, *Bam*HI, and *Bcl*I were subjected to electrophoresis on a 1% agarose gel. The DNA fragments were then transferred to Hybond-N filters. Each filter was then hybridized separately at 45°C with ^{32}P-labelled JLS16 in hybridization buffer containing (A) 20%, (B) 30%, (C) 40%, and (D) 50% formamide. The membranes were washed twice for 30 minutes in 2X SSC, 0.1% SDS solution and twice for 30 minutes in 0.2X SSC, 0.1% SDS solution. Size markers are shown in kb.

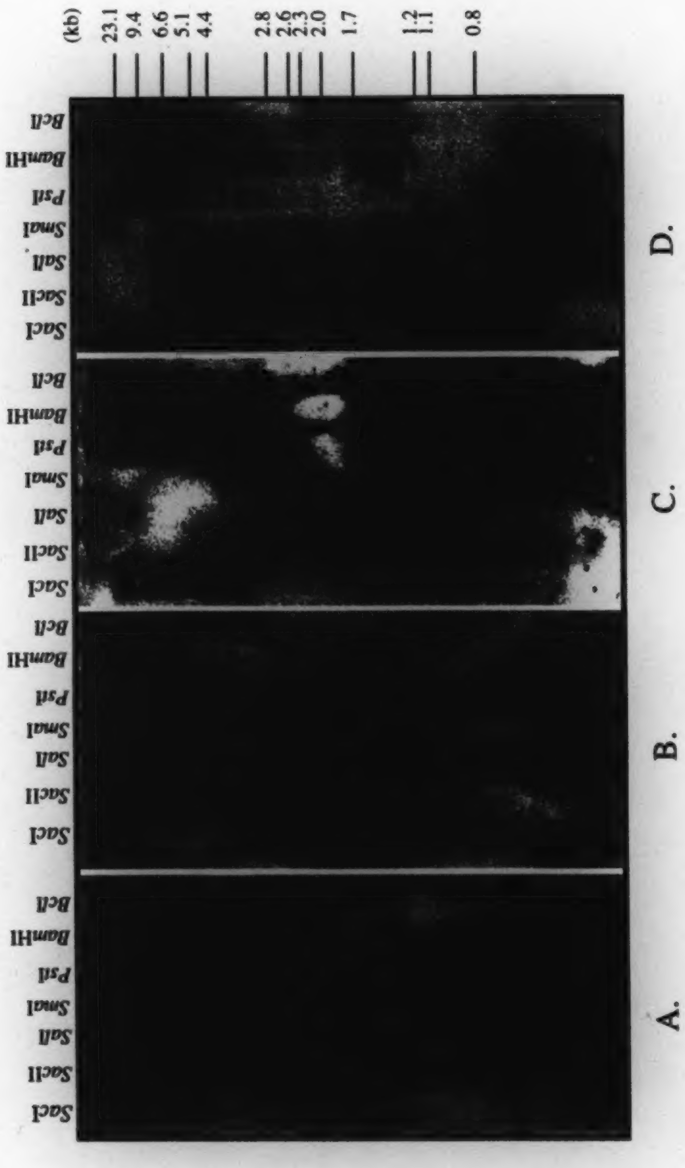
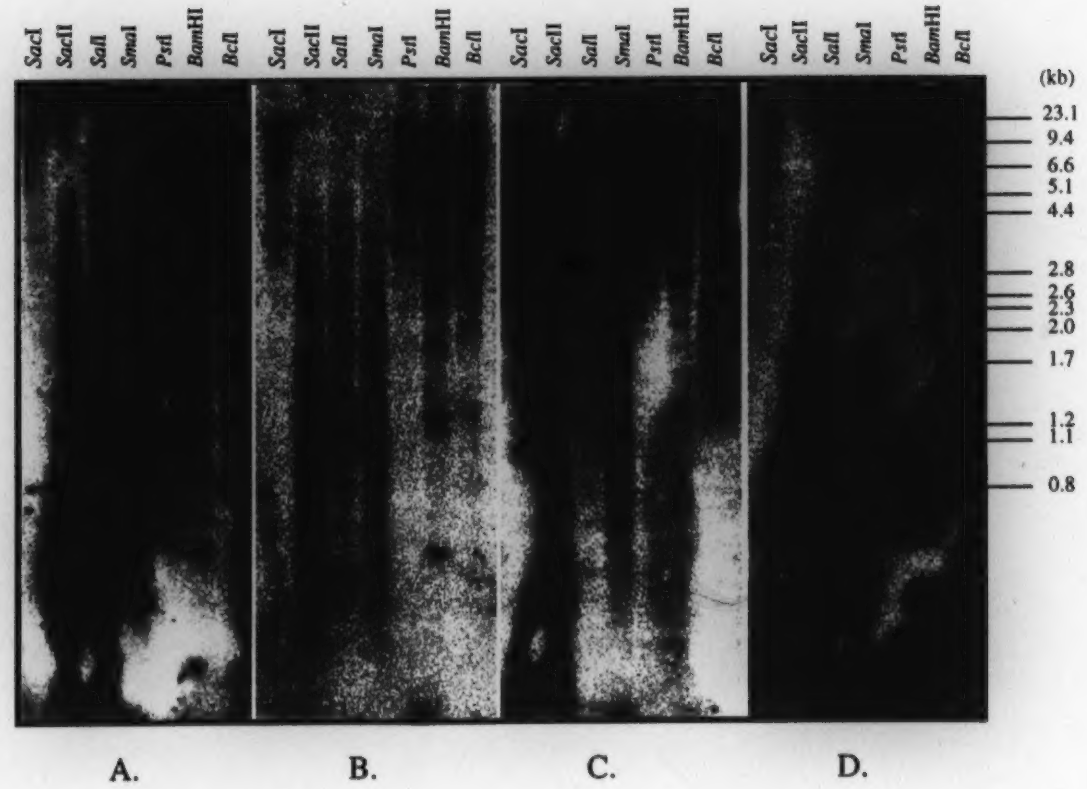

(kb)
23.1
9.4
6.6
5.1
4.4
2.8
2.6
2.3
2.0
1.7
1.2
1.1
0.8
SacI
SacII
SalI
SmaI
PstI
BamHI
BclI
A.
B.
C.
D.

Plate III.9.3.2. Autoradiograms of Southern transfers of restriction enzyme digested *S. coelicolor* J1501 genomic DNA probed with JLS17 in the presence of formamide. Four sets of seven identical aliquots of *S. coelicolor* DNA digested with *Sac*I, *Sac*II, *Sal*I, *Sma*I, *Pst*I, *Bam*HI, and *Bcl*I were subjected to electrophoresis on a 1% agarose gel. The DNA fragments were then transferred to Hybond-N filters. Each filter was then hybridized separately at 45°C with ^{32}P-labelled JLS17 in hybridization buffer containing (A) 20%, (B) 30%, (C) 40%, and (D) 50% formamide. The membranes were washed twice for 30 minutes in 2X SSC, 0.1% SDS solution and twice for 30 minutes in 0.2X SSC, 0.1% SDS solution. Size markers are shown in kb.


SacI
SacII
SalI
SmaI
PstI
BamHI
BclI
SacI
SacII
SalI
SmaI
PstI
BamHI
BclI
SacI
SacII
SalI
SmaI
PstI
BamHI
BclI
SacI
SacII
SalI
SmaI
PstI
BamHI
BclI
(kb)
23.1
9.4
6.6
5.1
4.4
2.8
2.6
2.3
2.0
1.7
1.2
1.1
0.8
A.
B.
C.
D.

Since multiple bands were still present in some of the lanes, it was thought that more stringent conditions were necessary, therefore, the membranes were also washed with solutions containing formamide (Plate III.9.3.3 and Plate III.9.3.4). This more stringent wash did result in cleaner, less ambiguous results but there was still multiple bands present indicating that the stringency was not yet high enough. The Southerns were washed with even more stringent conditions by adding a final wash with 0.1X SSC, 0.1% SDS, X% formamide, where X is the percentage of formamide present in the hybridization buffer (Plate III.9.3.5). The more stringent wash conditions improved the background hybridization but multiple bands were still observed. To ensure that these bands were not due to incomplete digestion, the experiment was repeated, this time increasing the amount of restriction endonuclease used (Plate III.9.3.6). The number of multiple bands was reduced. Based on these results, a 1.1 kb *Sma*I fragment and the 2.4 kb and 1.8 kb *Sac*II fragments were chosen for further cloning experiments.

III.9.4 Cloning of the Hybridizing Fragments —Trial#2

Based on the hybridization of the 50-mer oligonucleotide probe, JLS16, to *S. coelicolor* J1501 genomic digests, three hybridizing fragments were chosen for cloning experiments: a *Sma*I fragment of 1.1 kb and two *Sac*II fragments of 2.4 kb and 1.8 kb.

S. coelicolor J1501 chromosomal DNA was digested with *Sac*II and fragments in the size range of 1.8 kb and 2.4 kb were gel-purified by the trough method (Zhen and Swank, 1993) and hybridization of the purified fraction to JLS16 under stringent conditions was confirmed by Southern analysis (not shown). The fragments were

Plate III.9.3.3. Effect of formamide wash solutions on the autoradiograms of Southern transfers of restriction enzyme digested *S. coelicolor* J1501 genomic DNA probed with JLS16 in the presence of formamide. Four sets of seven identical aliquots of *S. coelicolor* DNA digested with *Sac*I, *Sac*II, *Sal*I, *Sma*I, *Pst*I, *Bam*HI, and *Bcl*I were subjected to electrophoresis on a 1% agarose gel. The DNA fragments were then transferred to Hybond-N filters. Each filter was then hybridized separately at 45°C with ^{32}P-labelled JLS16 in hybridization buffer containing (A) 20%, (B) 30%, (C) 40%, and (D) 50% formamide. The membranes were washed with solutions containing formamide equivalent to the amount present during hybridization: twice for 30 minutes in 2X SSC, 0.1% SDS solution, X% formamide, twice for 30 minutes in 0.2X SSC, 0.1% SDS, X% formamide solution. Size markers are shown in kb.

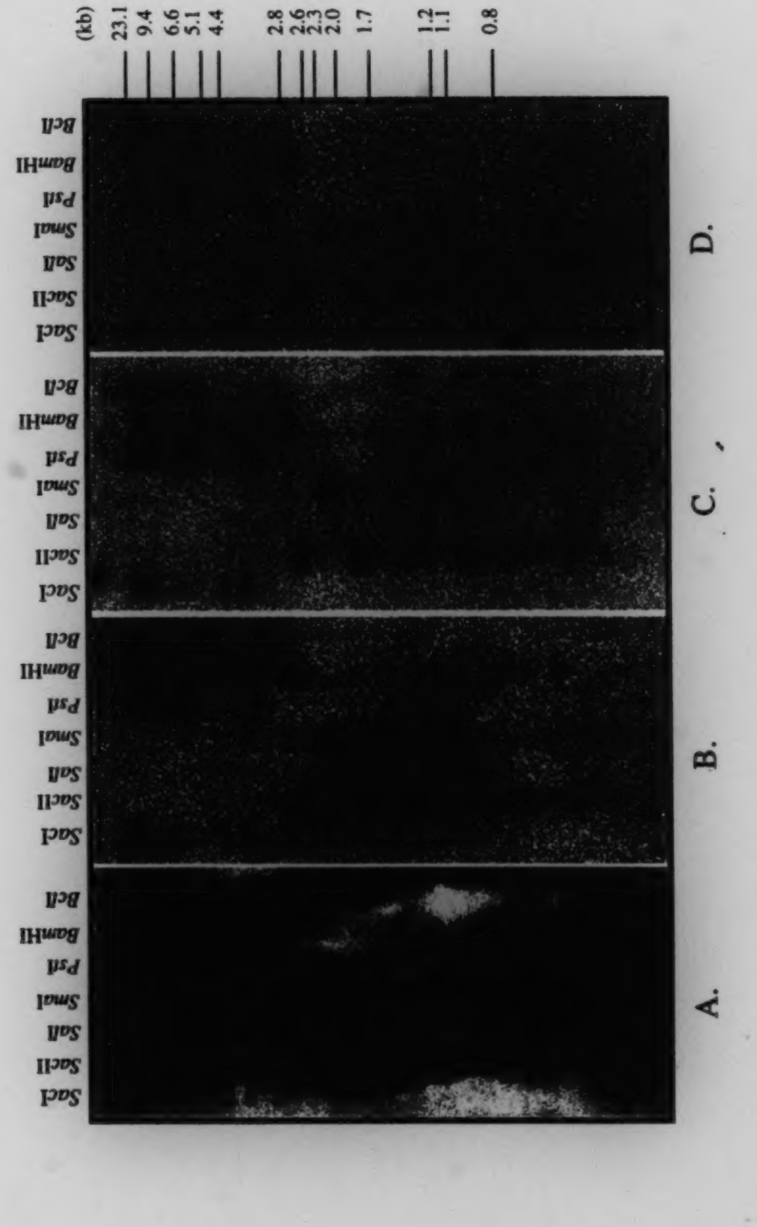
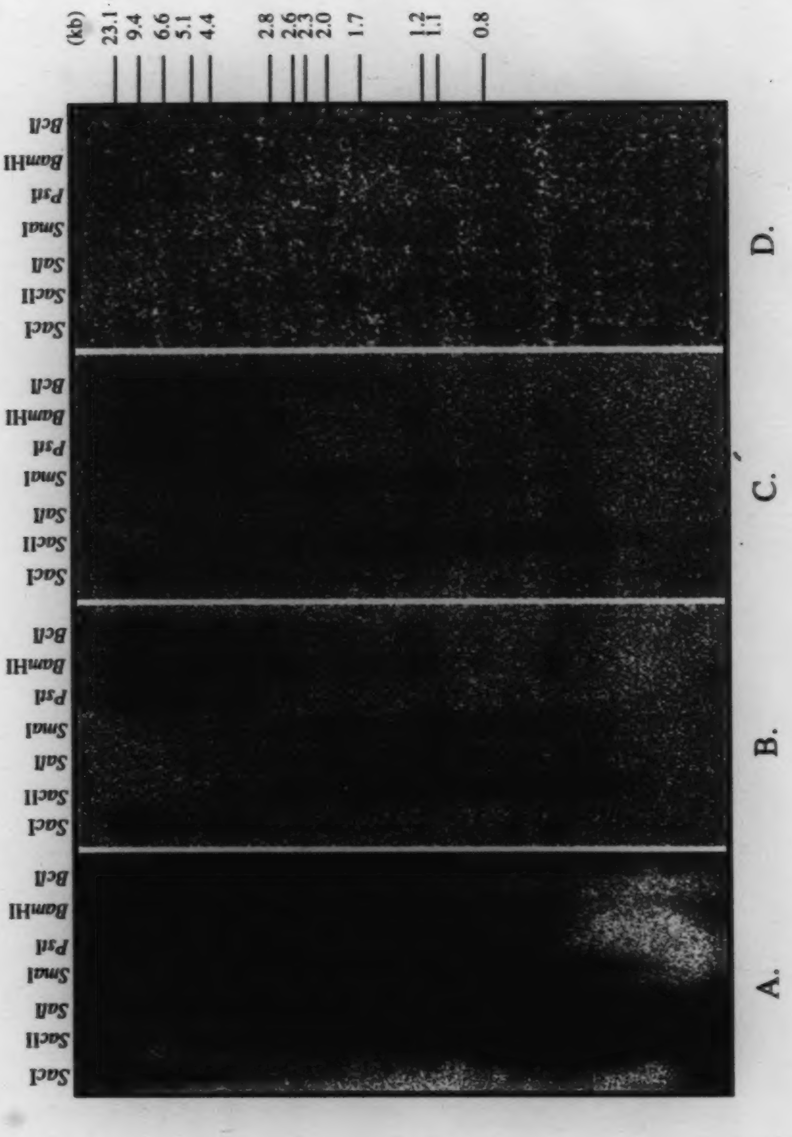

(kb)
23.1
9.4
6.6
5.1
4.4
2.8
2.6
2.3
2.0
1.7
1.2
1.1
0.8
SacI
SacII
SalI
SmaI
PstI
BamHI
BclI
SacI
SacII
SalI
SmaI
PstI
BamHI
BclI
SacI
SacII
SalI
SmaI
PstI
BamHI
BclI
SacI
SacII
SalI
SmaI
PstI
BamHI
BclI
A.
B.
C.
D.

Plate III.9.3.4. Effect of formamide wash solutions on the autoradiograms of Southern transfers of restriction enzyme digested *S. coelicolor* J1501 genomic DNA probed with JLS17 in the presence of formamide. Four sets of seven identical aliquots of *S. coelicolor* DNA digested with *Sac*I, *Sac*II, *Sal*I, *Sma*I, *Pst*I, *Bam*HI, and *Bcl*I were subjected to electrophoresis on a 1% agarose gel. The DNA fragments were then transferred to Hybond-N filters. Each filter was then hybridized separately at 45°C with ^{32}P-labelled JLS17 in hybridization buffer containing (A) 20%, (B) 30%, (C) 40%, and (D) 50% formamide. The membranes were washed with solutions containing formamide equivalent to the amount present during hybridization: twice for 30 minutes in 2X SSC, 0.1% SDS solution, X% formamide, twice for 30 minutes in 0.2X SSC, 0.1% SDS, X% formamide solution. Size markers are shown in kb.



(kb)
23.1
9.4
6.6
5.1
4.4
2.8
2.6
2.3
2.0
1.7
1.2
1.1
0.8
SacI
SacII
SalI
SmaI
PstI
BamHI
BclI
A.
B.
C.
D.

Plate III.9.3.5. Effect of stringent washes on the autoradiograms of Southern transfers of restriction enzyme digested *S. coelicolor* J1501 genomic DNA probed with JLS16 and JLS17 in the presence of formamide. Three sets of seven identical aliquots of *S. coelicolor* DNA digested with *Sac*I, *Sac*II, *Sal*I, *Sma*I, *Pst*I, *Bam*HI, and *Bcl*I were subjected to electrophoresis on a 1% agarose gel. The DNA fragments were then transferred to Hybond-N filters. Each filter was then hybridized separately at 45°C with ^{32}P-labelled JLS16 or ^{32}P-labelled JLS17 in hybridization buffer containing 30%, 40%, and 50% formamide. The membranes were washed with solutions containing formamide equivalent to the amount (X) present during hybridization: twice for 30 minutes in 2X SSC, 0.1% SDS solution, X% formamide, twice for 30 minutes in 0.2X SSC, 0.1% SDS, X% formamide solution and twice with 0.1X SSC, 0.1% SDS for 30 minutes. Size markers are shown in kb.

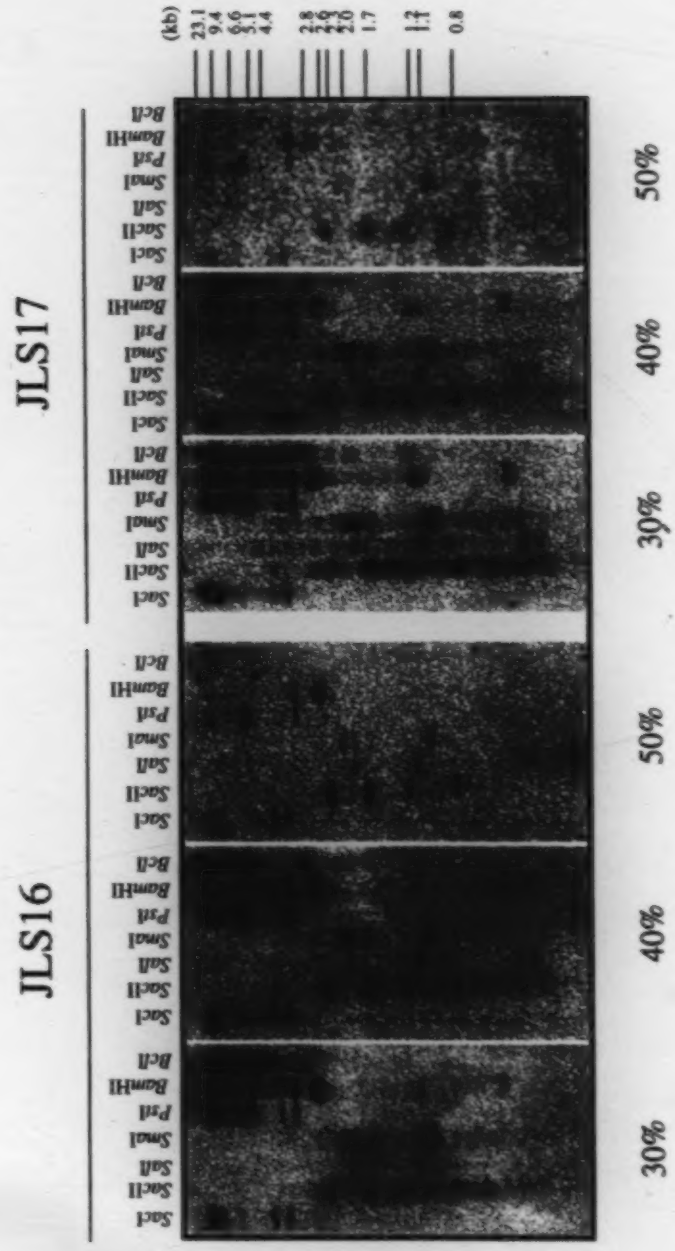
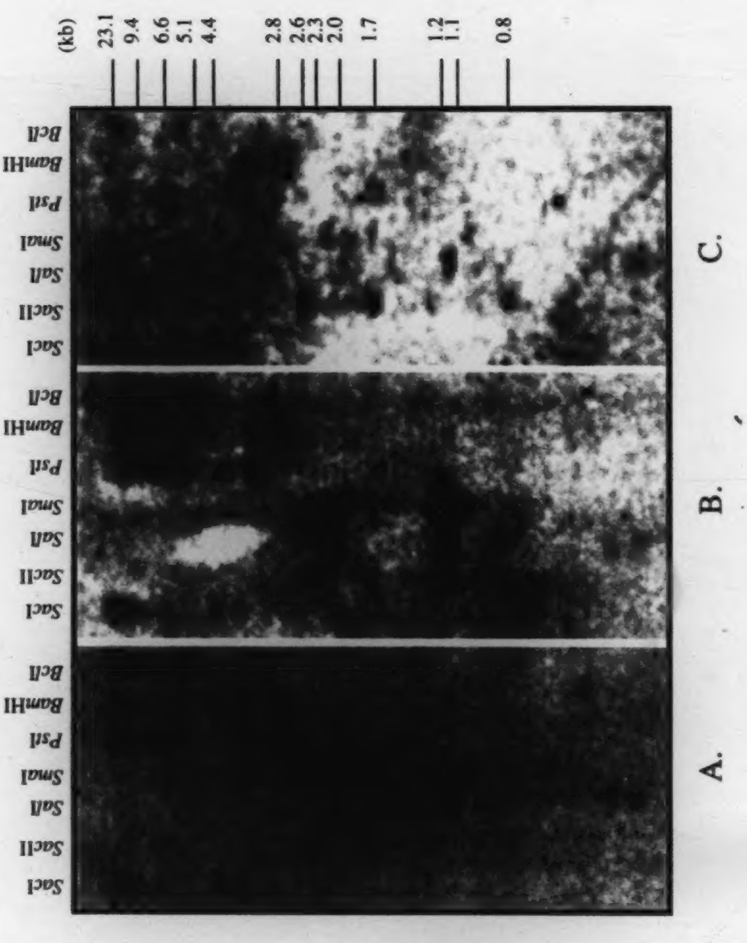

JLS16
JLS17
SacI
SacII
SalI
SmaI
PstI
BamHI
BclI
30%
40%
50%
(kb)
23.1
9.4
6.6
5.1
4.4
2.8
2.6
2.3
2.0
1.7
1.2
1.1
0.8

Plate III.9.3.6. Autoradiogram of Southern transfers of enhanced restriction enzyme digested *S. coelicolor* J1501 genomic DNA probed with JLS16 in the presence of formamide. Three sets of seven identical aliquots of *S. coelicolor* DNA digested with *Sac*I, *Sac*II, *Sal*I, *Sma*I, *Pst*I, *Bam*HI, and *Bcl*I were subjected to electrophoresis on a 1% agarose gel. The DNA fragments were then transferred to Hybond-N filters. Each filter was then hybridized separately at 45°C with ^{32}P-labelled JLS16 in hybridization buffer containing (A) 30%, (B) 40%, and (C) 50% formamide. The membranes were washed with solutions containing formamide equivalent to the amount (X) present during hybridization: twice for 30 minutes in 2X SSC, 0.1% SDS solution, X% formamide, twice for 30 minutes in 0.2X SSC, 0.1% SDS, X% formamide solution and twice with 0.1X SSC, 0.1% SDS for 30 minutes. Size markers are shown in kb.


(kb)
23.1
9.4
6.6
5.1
4.4
2.8
2.6
2.3
2.0
1.7
1.2
1.1
0.8
SacI
SacII
SalI
SmaI
PstI
BamHI
BclI
A.
B.
C.

shotgun cloned into *Sst*II-digested pBluescript KS$^+$ and transformed into *E. coli* DH5α. When white, insert-containing clones were subcultured for screening, none of them were viable. Attempts to clone these *Sac*II fragments into pBluescript SK$^+$ so that they would be in the opposite orientation with respect to vector sequences also failed. Attempts to shotgun clone the blunted *Sac*II fragments into the *Sma*I site of the multiple cloning site (MCS) of pUC119 also failed. In this case, although twenty-seven transformants were obtained, none of them hybridized to the JLS16 or JLS17 probes.

In an attempt to clone the 1.1 kb *Sma*I fragment, *S. coelicolor* J1501 chromosomal DNA was digested with *Xma*I (*Xma*I recognizes the same nucleotide sequence as *Sma*I but generates 5' overhangs instead of blunt ends) and fragments in the 1.1 kb range were gel-purified by the trough method. An aliquot of the purified 1.1 kb fraction was transferred to Hybond-N membrane and probed with JLS16 to confirm that the population of purified DNA contained a JLS16-hybridizing fragment. The 1.1 kb fragment was cloned into the *Xma*I site of the pUC119 multiple cloning site and transformed into *E. coli* DH5α. This time the LB culture plates were supplemented with ampicillin and 0.4% glucose. Glucose was added in an attempt to stabilize the clones since the *lac* promoter which controls the *lacZ'* gene fragment of pUC based vectors should be repressed under these conditions. Also, since the *lac* promoter is induced by IPTG, which was not added to the medium, transcription of any gene inserted downstream of it should not occur. Only eighteen transformants were obtained. The DNA was isolated and digested with *Xma*I, separated on a 1% agarose gel and transferred to a Hybond-N membrane. The Southern blot was probed with [γ-^{32}P] ATP end-labelled

JLS16. Only one clone hybridized and was designated 45-37 (Plate III.9.4.1). Double-stranded DNA of this plasmid was sent for sequence analysis (Department of Biological Sciences Sequencing Service, University of Alberta) using JLS16 as well as forward and reverse universal sequencing primers. Enough sequence of this clone was obtained to perform a database search. Sequence of this plasmid, p45-37, did not reveal the oligonucleotide sequence of JLS16 nor was the amino acid sequence JLS16 represented in any of the six translation frames. A search of the Sanger Centre database placed this stretch of DNA on cosmid SC5F2A. Because of the negative result, the cloning procedure was repeated. This time only seven white transformants were obtained. Southern analysis of *Xma*I digested plasmid DNA from the transformants using [γ-^{32}P] ATP end-labelled JLS16, revealed that four of the plasmids contained hybridizing inserts (Plate III.9.4.2). The plasmid, p58-22, appeared to have a double insert; this plasmid had the most intense hybridization signal so the plasmid was digested with *Xma*I and the inserts were gel-purified and subcloned into pUC119 (Plate III.9.4.3) in an attempt to obtain clones with single inserts. Fifty of the potentially positive subclones were screened by hybridization with end-labelled JLS16 (Plate III.9.4.3) and 24 were found to be positive. Three were chosen for sequencing and designated p64-44, p64-17, and p64-25. Forward and reverse primers were used for sequence determination. The entire clone was not sequenced but enough sequence was obtained to search the *S. coelicolor* database. Sequence analysis of these three plasmids demonstrated that they all possessed the same insert DNA (Table II.2.1.3) but did not possess the oligonucleotide sequence that was used as probe. The DNA and translated sequences were searched against the Sanger Centre database. The DNA sequence matched cosmid SCF11 and the

Plate III.9.4.1. Restriction and Southern analysis of *Xma*I (1.1 kb) clones. DNA extracted from transformants arising from the ligation of the 1.1 kb *Xma*I digested and purified DNA (which hybridized to JLS16) into pUC119 were digested with *Xma*I and separated by electrophoresis on a 1% agarose gel. (A) The gel was stained in ethidium bromide and visualized by exposure to long wave ultraviolet light on a transilluminator. (B) The DNA fragments were transferred to a Hybon-N membrane and probed with end-labelled JLS16 in 40% formamide at 45°C. The membrane was washed with 2X SSC, 0.1% SDS, 40% formamide for 30 minutes and with 0.2X SSC, 0.1%SDS, 40% formamide for an additional 30 minutes and exposed overnight to a phosphorimager screen. Letters 'a' through 'r' represent different transformants. Clone 'c' was later designated 45-37.

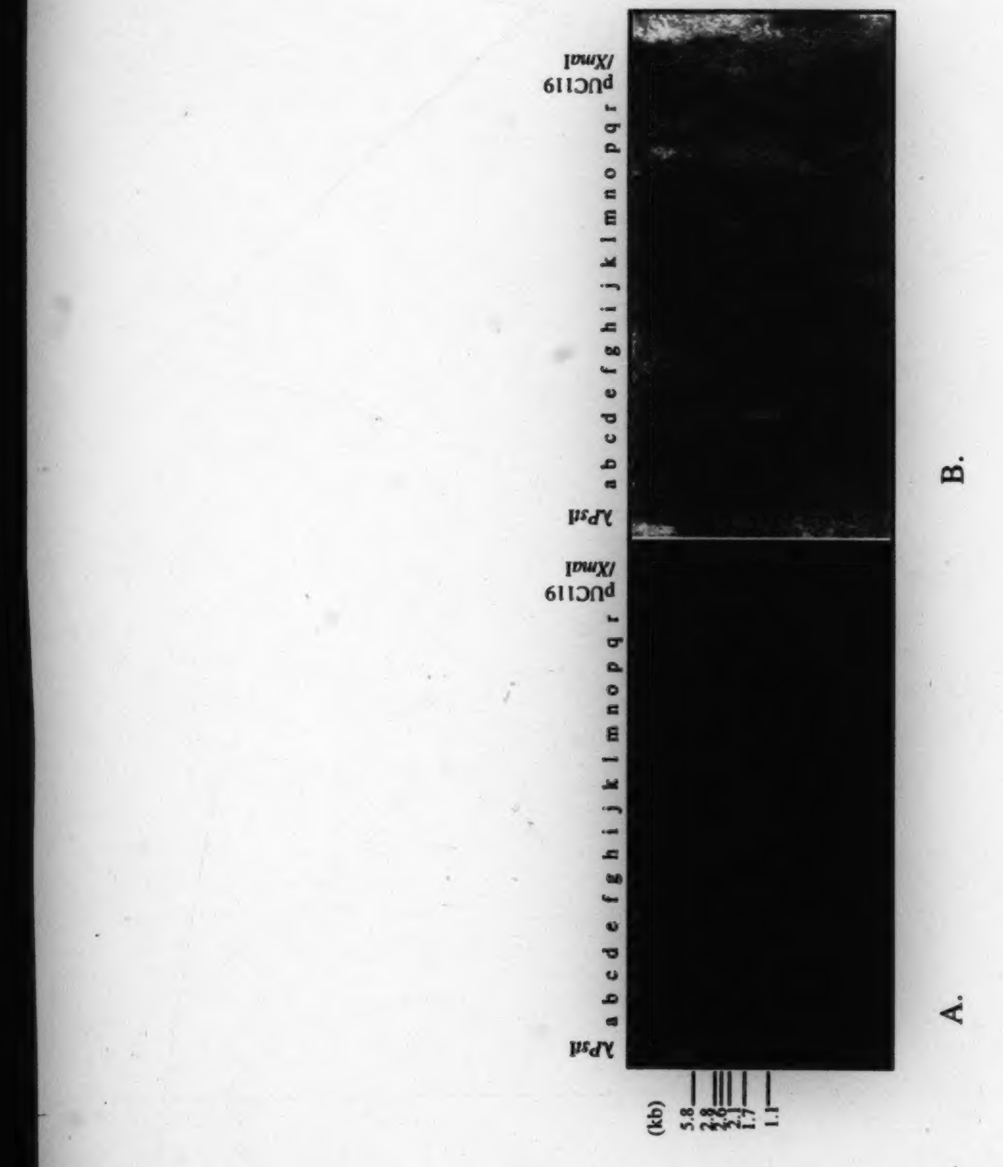

λPstI
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
pUC119
/XmaI
A.
λPstI
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
pUC119
/XmaI
B.
(kb)
5.8
2.8
2.6
2.1
1.7
1.1

Plate III.9.4.2. Restriction and Southern analysis of clones generated by cloning a 1.1 kb *Xma*I fragment into pUC119. Plasmid DNA was digested with *Xma*I and separated by electrophoresis on a 1% agarose gel. (A) The gel was stained in ethidium bromide and visualized by exposure to long wave ultraviolet light on a transilluminator. (B) The DNA fragments were transferred to a Hybond-N membrane and probed with JLS16 in 40% formamide at 45°C. The membrane was washed with 2X SSC, 0.1% SDS, 40% formamide for 30 minutes and with 0.2X SSC, 0.1%SDS, 40% formamide for an additional 30 minutes and exposed overnight to a phosphorimager screen. (1) λ*Hin*dIII, (2) λ*Pst*I, (3) p59-17, (4) p59-35, (5) p59-41, (6) p59-42, (7) p59-47, (8) p59-50, (9) p58-22.

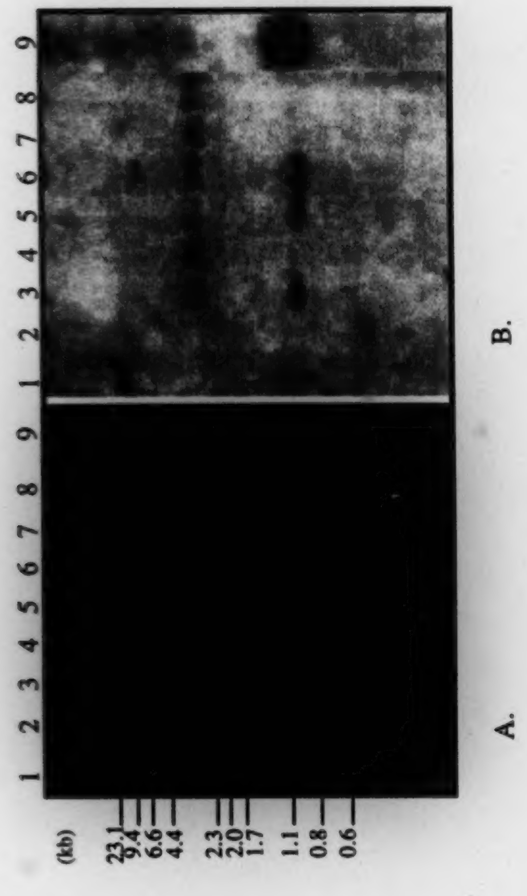
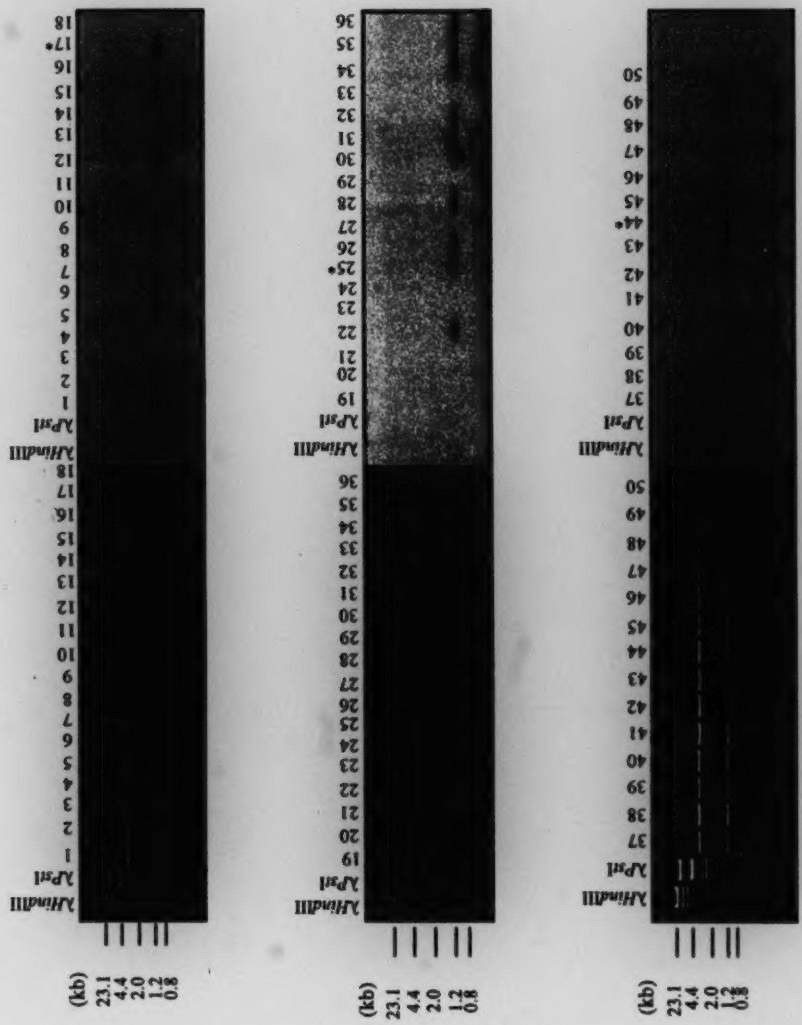

(kb)
23.1
9.4
6.6
4.4
2.3
2.0
1.7
1.1
0.8
0.6
1 2 3 4 5 6 7 8 9
A.
1 2 3 4 5 6 7 8 9
B.

Plate III.9.4.3. Restriction and Southern analysis of subclones generated from p58-22. Plasmid DNA was digested with *Xma*I and separated by electrophoresis on a 1% agarose gel. (A) The gel was stained in ethidium bromide and visualized by exposure to long wave ultraviolet light on a transilluminator. (B) The DNA fragments were transferred to a Hybon-N membrane and probed with JLS16 in 40% formamide at 45°C. The membrane was washed with 2X SSC, 0.1% SDS, 40% formamide for 30 minutes and with 0.2X SSC, 0.1%SDS, 40% formamide for an additional 30 minutes and exposed to a phosphorimager screen overnight. Numbers 1 through 50 represent plasmid DNA from different subclones.

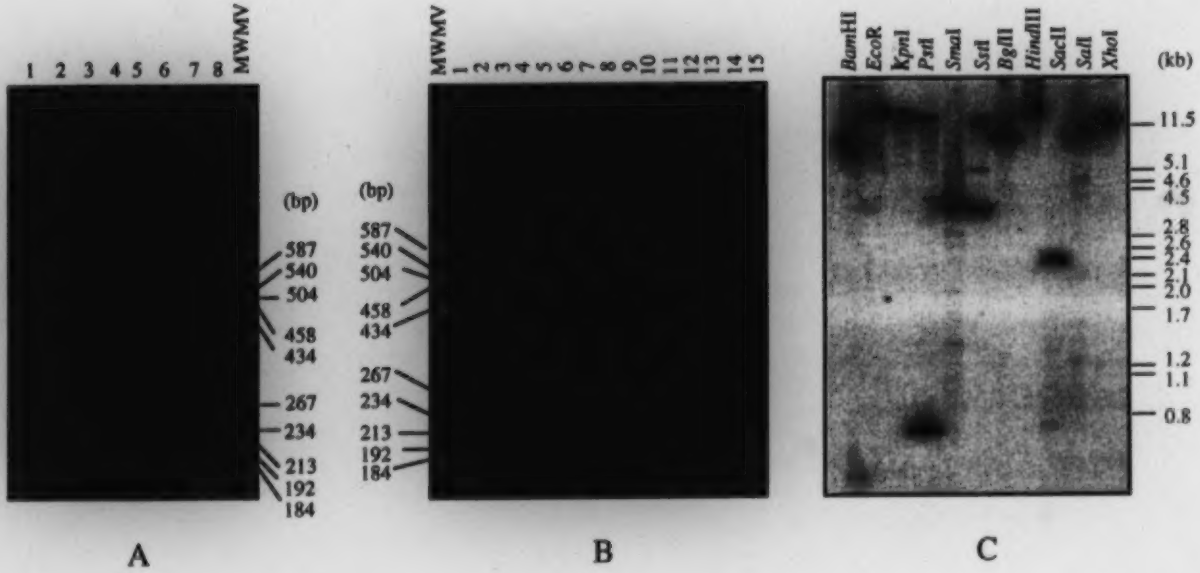

λHindIII
λPstI
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17*
18
λHindIII
λPstI
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
(kb)
23.1
4.4
2.0
1.2
0.8
λHindIII
λPstI
19
20
21
22
23
24
25*
26
27
28
29
30
31
32
33
34
35
36
λHindIII
λPstI
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
(kb)
23.1
4.4
2.0
1.2
0.8
λHindIII
λPstI
37
38
39
40
41
42
43
44*
45
46
47
48
49
50
λHindIII
λPstI
37
38
39
40
41
42
43
44
45
46
47
48
49
50
(kb)
23.1
4.4
2.0
1.2
0.8

translated sequence showed homology to two proteins: SCF11.10c, which is a hypothetical regulator of 288 amino acids similar to an *E. coli* fatty acyl reponsive regulator with a *gntR* family signature, and SCF11.09c, which is a possible oxidoreductase of 508 amino acids similar to a *Zymomonas mobilis* glucose-fructose oxidoreductase precursor. The %GC of these two genes was found to be 73% and 72% respectively. The amino acid sequences did not show homology to the internal peptide sequences used to design the oligonucleotide probes used for these experiments. Thus despite stringent hybridization conditions, false positives were obtained.

III.10 Amplification of the *sucD* Gene by Polymerase Chain Reaction and Its Sequence Analysis

Since cloning proved to be problematic for the gene of the 32 kDa phosphorylated protein, alternative methods had to be employed so that this gene could be characterized. Since two internal amino acid sequences were obtained from peptide sequencing, PCR amplification of the gene was attempted. A prediction of the size of the product that would be obtained by PCR amplification was not possible since the proximity of the two peptide sequences was unknown. JLS2 (5'-ATGGG(AG)CACGC(CG)GG(CG)GC(GC)ATCGT-3') and its complementary sequence JLS10 (5'-TACCC(TC)GTGCG(GC)CC(GC)CG(CG)TAGCA-3') and JLS3 (5'-GG(GC)GAGGT(CG)GG(GC)AACGC(GC)GAGCAG-3') and its complementary sequence JLS11 (5'-CC(GC)CTCCA(GC)CC(CG)TTGCG(GC)CTCGTC-3') were used in PCR experiments (Plate III.10.1). PCR amplification with JLS2 and JLS11 revealed a product of approximately 600 bp (Plate III.10.1-A) while JLS3 and JLS10 did not produce a product (not shown). As expected, a product was not observed in the control reaction that did not contain

Plate III.10.1. Initial attempts to amplify the gene for the 32 kDa protein by polymerase chain reaction. **(A)** Representative 1% agarose gel of PCR amplification with the oligonucleotide primers JLS2 and JLS11. The complementary reaction with primers JLS10 and JLS3 did not yield any product and therefore gels of this reaction are not shown. Lanes 1,2,3: aliquots of separate reactions containing 1 µg *S. coelicolor* J1501 template DNA with primers JLS2 and JLS11. Lane 4: reaction results with both primers but no template DNA. Lanes 5,6, and 7: aliquots of separate reactions containing 0.5 µg *S. coelicolor* J1501 template DNA with primers JLS2 and JLS11. Lane 8: reaction with JLS11 primer alone. **(B)** Agarose gel of control reactions. (1) JLS2 and JLS11 + 0% DMSO. (2) JLS2 and JLS11 + 2% DMSO. (3) JLS2 and JLS11 + 4% DMSO. (4) JLS2 and JLS11 + 6% DMSO. (5) JLS2 and JLS11 + 8% DMSO. (6) JLS2 alone + 0% DMSO. (7) JLS11 alone + 0% DMSO. (8) JLS10 and JLS3 + 0% DMSO. (9) JLS10 and JLS3 + 2% DMSO. (10) JLS10 and JLS3 + 4% DMSO. (11) JLS10 and JLS3 + 6% DMSO. (12) JLS10 and JLS3 + 8% DMSO. (13) JLS10 alone + 0% DMSO. (14) JLS 3 alone + 0% DMSO. (15) No template DNA plus JLS2 and JLS11 + 0% DMSO. **(C)** Southern analysis of *S. coelicolor* J1501 digests probed with purified PCR product. Hybridization was performed in buffer containing 50% formamide at 65°C. Wash solutions contained 50% formamide.


1 2 3 4 5 6 7 8 MWMV
(bp)
587
540
504
458
434
267
234
213
192
184
A
MWMV 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
(bp)
587
540
504
458
434
267
234
213
192
184
B
BamHI
EcoR
KpnI
PstI
SmaI
SstI
BglII
HindIII
SacII
SalI
XhoI
(kb)
11.5
5.1
4.6
4.5
2.8
2.6
2.4
2.1
2.0
1.7
1.2
1.1
0.8
C

template DNA; nor was a product observed when JLS11 was the sole primer in the reaction. Unfortunately, a control reaction with primer JLS2 was not performed at the same time. Since there was only one amplified product obtained from these reactions, the 600 bp PCR product was gel-purified by the 'crush and soak' method (Sambrook *et al.*, 1989). The purified PCR product was used as a probe against digests of *S. coelicolor* J1501 genomic DNA (Plate III.10.1-C) and the amplified DNA was also sequenced (Department of Biological Sciences Sequencing Service, University of Alberta). Translation of the sequence obtained from this PCR product revealed the sequence MGHAGAIV (corresponding to JLS2) repeated throughout the length of the sequence, therefore this primer must have produced the amplification product alone. As mentioned above, a control of the JLS2 primer had been overlooked. To confirm that the JLS2 primer was able to generate an amplified product without the presence of a second primer, a set of control reactions was performed (Plate III.10.1-B). An amplification product was generated from JLS2 alone and therefore the product observed in the reactions with JLS2 and JLS11 was due to the single primer. JLS2 was obviously able to bind to more than one template sequence under the amplification conditions employed. The fact that no additional amplified bands were observed when the two primers were present in the reaction might suggest that they do not correspond to sequences present in a single gene. In light of these results, it was suspected that perhaps the two peptide sequences obtained were from two distinct proteins that had co-purified.

As stated earlier, database searches for homologous proteins to the two peptide sequences obtained from the 32 kDa phosphorylated protein were not initially informative. Periodic database searches were performed and finally revealed that the amino acid sequence, TMGHAGAIVSGSSGTAQAK, showed homology to the alpha subunit of succinyl-CoA synthetase (SCS) of *Mycobacterium tuberculosis* and *M. leprae* (Figure III.10.2). The second internal amino acid sequence, ATVGEVGNAEQSNINWGK, was found to be homologous to the 50S ribosomal protein, L2, of *M. leprae* and *M. bovis* (Figure III.10.3). This confirmed earlier suspicions that the peptide sequences obtained from protein sequencing were from separate proteins.

In light of these findings, it was decided that primers based on the *Mycobacterium* protein sequence would be used to amplify the homologous *sucD* gene from the *Streptomyces coelicolor* genome (see below for PCR of L2). Initial attempts at the amplification of the α-subunit of SCS using the degenerate primers JLS34 (5'-GG(GC)GTCTT(GC)CC(GC)AC(TC)TT(AGCT)AC-3') and JLS35 (5'-GACAACAAGGT(ACGT)AT(ACT)GT(ACGT)CA-3') based on the protein sequence of *M. tuberculosis* were not successful when standard PCR conditions were employed. The expected size of the amplification product was 836 bp. Optimization of the $MgCl_2$ concentration was attempted whereby $MgCl_2$ was added to the reactions in increments from 0 mM to 9 mM. Again, no product was observed, only a smear in the lane for reactions containing 0 mM and absolutely nothing in the lanes for reactions containing additional $MgCl_2$. Additional trials at optimization were attempted in which template, DMSO, dNTPs and primer concentration as well as annealing and extension temperatures were varied in separate experiments but still no product was observed. The PCR_x Enhancer System (GIBCO-BRL) was also tried but no amplification was observed. Finally, the Actinomycete PCR protocol described by Eung-Soo Kim (http://molbio.cbs.umn.edu/asirc/protocol/pcrhighgc.html) was attempted. This protocol

Figure III.10.2. BLAST search result of a query for homologous proteins to internal peptide sequence 1. Peptide sequence 1 was obtained from protein sequence analysis of the 32 kDa phosphorylated protein.

SUCCINYL-COA SYNTHETASE ALPHA CHAIN (*sucD*)

```
Peptide seq1:       1   TMGHAGAIVSGSSGTAQAK 19
                        TMGHAGAIVSGSSGTA AK
M.tuberculosis:   256   TMGHAGAIVSGSSGTAAAK 274
```

```
Peptide seq1:       1   TMGHAGAIVSGSSGTAQAK 19
                        TMGHAGAIVSGSSGTA  K
M. leprae:         253  TMGHAGAIVSGSSGTAAVK 271
```

Figure III.10.3. BLAST search result of a query for homologous proteins to internal peptide sequence 2. Peptide sequence 2 was obtained from protein sequence analysis of the 32 kDa phosphorylated protein.

50S RIBOSOMAL PROTEIN L2 (*rplB*)

```
Peptide seq2:   1   ATVGEVGNAEQSNINWGK 18
                    ATVGEVGNAEQ+NINWGK
M. leprae:      191 ATVGEVGNAEQANINWGK 208
```

```
Peptide seq2:   1   ATVGEVGNAEQSNINWGK 18
                    ATVGEVGNAEQ+NINWGK
M. bovis:       191 ATVGEVGNAEQANINWGK 208
```

used a very low annealing temperature and a lengthy extension time (Plate III.10.4). DMSO and $MgCl_2$ concentrations were optimized. As expected, neither primer alone was able to amplify the 836 bp product. Even though additional bands were observed, the amplified products generated by the controls were used to distinguish the desired product at 836 bp. The 836 bp PCR product generated by amplification from the *S. coelicolor* J1501 chromosome by primers JLS34 and JLS35 was gel-purified and used as template for sequencing (Department of Biological Sciences Sequencing Service, University of Alberta). The peptide sequence corresponding to TMGHAGAIVSGSSGTAQAK was identified upon translation of the sequence thus confirming that this PCR product did indeed correspond to the DNA sequence of the *sucD* gene. The sequence was searched against the Sanger Centre Database for *Streptomyces* and was found to be on cosmid SC8A6. The 836 bp PCR product represented an internal region of the *sucD* gene. The cosmid sequence from the Sanger Centre database was used to construct PCR primers so that the termini of the *sucD* gene could be sequenced since attempts at inverse PCR were not successful. Primers were constructed such that the two PCR products generated would span the entire gene sequence. This enabled the confirmation of previous sequence data and made it so that each strand was sequenced twice. To determine if there were any differences in DNA sequence of the *sucD* gene between the wild type strain and the mutant strain, J1501 and J1681 were both used as template for amplification. The entire nucleotide sequence of the *sucD* gene and its translation to amino acids is shown in Figure III.10.5. No differences in the DNA sequence between J1501 and J1681 were found. The DNA sequence was a 100% match to the *sucD* gene sequence on cosmid SC8A6 that resulted

Plate III.10.4. Amplification of the *sucD* gene from the *S. coelicolor* genome by polymerase chain reaction. The Actinomycete PCR protocol was used to amplify the gene encoding the alpha subunit of the succinyl CoA synthetase 32 kDa phosphorylated protein. (A) Aliquots of PCR reactions were separated on a 1% agarose gel. (1) JLS34 primer alone + 5% DMSO (2) JLS35 primer alone + 5% DMSO (3) JLS34 + JLS35 + 10% DMSO (4) JLS34 + JLS35 + 10% DMSO (5) JLS34 + JLS35 + 5% DMSO (6) JLS34 + JLS35 + 5% DMSO + 1.5 mM $MgCl_2$. (B) Hot start PCR amplification of the *sucD* gene by the Actinomycete protocol. Reactions in lanes 1 through 6 contained 1.5 mM $MgCl_2$ and increasing concentrations of DMSO: (1) 5% (2) 6% (3) 7% (4) 8% (5) 9% (6) 10% (7) JLS34 alone + 5% DMSO (8) JLS35 alone + 5% DMSO. Reactions in lanes 9 through 16 contained 5% DMSO and the concentration of $MgCl_2$ was varied: (9) 1.5 mM (10) 2.5 mM (11) 3.5 mM (12) 4.5 mM (13) 5.5 mM (14) 6.5 mM (15) JLS34 alone + 1.5 mM $MgCl_2$ (16) JLS35 alone + 1.5 mM $MgCl_2$.

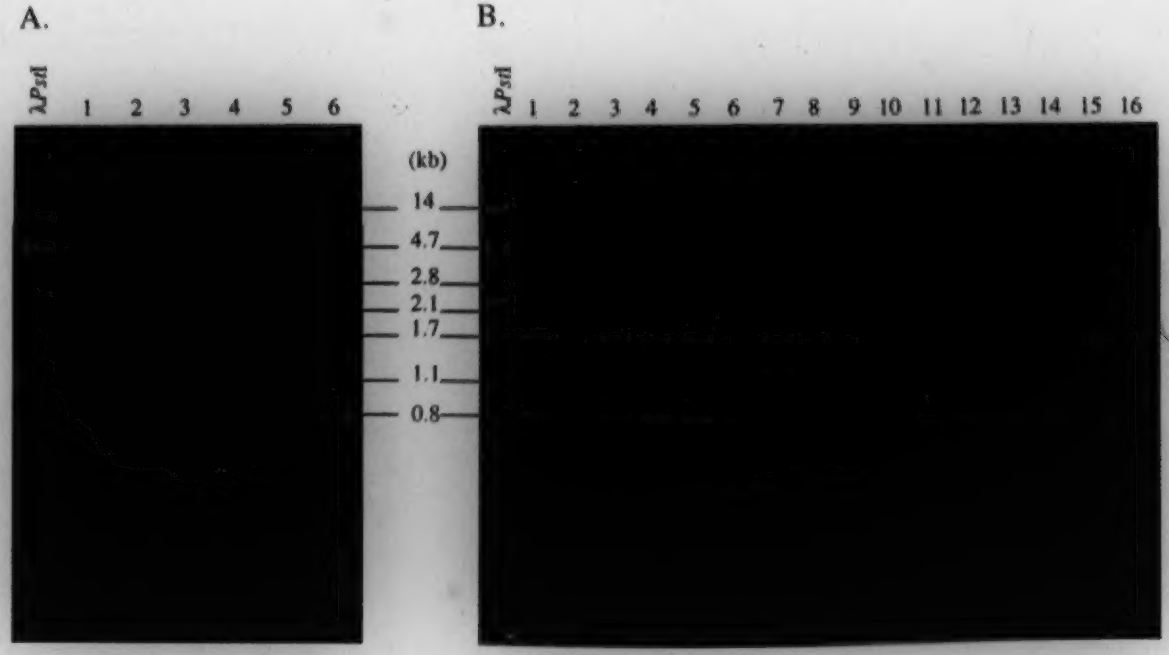

A.
λPstI
1
2
3
4
5
6
B.
λPstI
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
(kb)
14
4.7
2.8
2.1
1.7
1.1
0.8

Figure III.10.5. Nucleotide sequence and predicted amino acid sequence of the gene encoding the alpha subunit (*sucD*) of succinyl-CoA synthetase. The sequence from the end of *sucC* and the sequence of *sucD* from the translation start site to the termination codon are shown with the predicted amino acid sequence given directly below. The translation stop of *sucC* is shown (▨). The one letter amino acid code was used. The (*) denotes a stop codon. The numbers at the ends of the lines refer to base pair locations relative to the first base of the translation initiation codon. The peptide sequence obtained from microsequencing the 32 kDa-phosphorylated protein is boxed. The active site is underlined. The histidinyl residue that has been shown to be phosphorylated as part of the catalytic cycle in *E. coli* (Bridger, 1971) is shown with the (•).

```
                                                            CGACGC
      CCGCGCGCACCCCCTGGTCGAACAGGCCACCACCATGGACGGCGCCGCCCGCCGTGCCGC
                                              ▨
      CCGGCTCGCCACCGCGGCGTCCACCGCCGGACAGGCCGGATAAGGAGACAGGACGACATG
  1 - ATGGCGATCTACCTCACCAAGGAGAGCAAGGTCCTCGTCCAGGGCATGACCGGTGCCGAG - 60
    - M  A  I  Y  L  T  K  E  S  K  V  L  V  Q  G  M  T  G  A  E
 61 - GGCATGAAGCACACCCGCCGCATGCTGGCCGCGGGCACCGACGTCGTCGGCGGCGTCAAC - 120
    - G  M  K  H  T  R  R  M  L  A  A  G  T  D  V  V  G  G  V  N
121 - CCGCGCAAGGCGGGCCGCACCGTGGACTTCGACAACCGCACCGTCCCGGTCTTCGGATCG - 180
    - P  R  K  A  G  R  T  V  D  F  D  N  R  T  V  P  V  F  G  S
181 - GTCCGCGAGGGCATCGAACGCACGGGCGCCGACGTCACGGTCGTCTTCGTGCCACCGGCC - 240
    - V  R  E  G  I  E  R  T  G  A  D  V  T  V  V  F  V  P  P  A
241 - TTCGCCGAGGCCGCGGTCGTCGAGGCCGCCGACGCCGGGGTCGGCCTCGCGGTCGTCATC - 300
    - F  A  E  A  A  V  V  E  A  A  D  A  G  V  G  L  A  V  V  I
301 - ACCGAGGGCATCCCGGTCCACGACTCCGTCGCCCTCACCGCCCACGCAAGGGCCAAGGGC - 360
    - T  E  G  I  P  V  H  D  S  V  A  L  T  A  H  A  R  A  K  G
361 - ACCCGCGTCATCGGCCCCAACTGCCCCGGCCTGATCACCCCCGGCCAGTCCAACGCGGGC - 420
    - T  R  V  I  G  P  N  C  P  G  L  I  T  P  G  Q  S  N  A  G
421 - ATCATCCCGCCCGACATCACCAAGCCCGGCCGCATCGGCCTGGTCTCCAAGTCGGGCACG - 480
    - I  I  P  P  D  I  T  K  P  G  R  I  G  L  V  S  K  S  G  T
481 - CTCACCTACCAACTCATGTACGAACTGCGCGACATCGGCTTCTCCACCTGCGTCGGCATC - 540
    - L  T  Y  Q  L  M  Y  E  L  R  D  I  G  F  S  T  C  V  G  I
541 - GGCGGCGACCCGGTCGTCGGCACCAGCCACATCGACTGCCTCGCCGCCTTCGAGGACGAT - 600
    - G  G  D  P  V  V  G  T  S  H  I  D  C  L  A  A  F  E  D  D
601 - CCCGACACCGAACTGATCGTTCTCATCGGTGAGATCGGCGGCGACGCGGAGGAACGCGCG - 660
    - P  D  T  E  L  I  V  L  I  G  E  I  G  G  D  A  E  E  R  A
661 - GCGGCCCACATCCGCGCGCACGTCACCAAGCCCGTCGTCGCCTACATCGCCGGCTTCACC - 720
    - A  A  H  I  R  A  H  V  T  K  P  V  V  A  Y  I  A  G  F  T
721 - GCGCCCGAGGGCCGGACGATGGGCCACGCCGGTGCCATCGTCTCCGGCTCCTCGGGCACG - 780
    - A  P  E  G  R  [redacted]
781 - GCGCGGGCGAAGAAGGAGGCGCTGGAGTCGGTCGGCGTACGGGTCGGGAGCACACCGACC - 840
    - [redacted]     K  E  A  L  E  S  V  G  V  R  V  G  S  T  P  T
841 - GAGACGGCACGGCACGTACTCCAGGCACTCGACGTACCCGGAGGTGCCGCACCGGACGGC - 900
    - E  T  A  R  H  V  L  Q  A  L  D  V  P  G  G  A  A  P  D  G
901 - GCGGCCCGCGACGGAGCCCGCGCATGA - 927
    - A  A  R  D  G  A  R  A  *
```

from the genome sequencing project. A diagrammatic representation of the DNA surrounding the *sucD* gene is shown in Figure III.10.6. The gene encoding the β-subunit of SCS, *sucC*, lies directly upstream of the *sucD* gene. What is interesting is that there is a probable dehydrogenase (SC8A6.08) that overlaps *sucD* which probably has regulatory implications since *sucC*, *sucD* and *SC8A6.08* are probably transcribed as an operon (see Discussion).

Analysis of the nucleotide sequence for frame-shift sequencing errors was performed using the web-based FramePlot 2.3 program (http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl) (Ishikawa and Hotta, 1999) based on the Frame program designed by Bibb *et al.* (1984). Frame analysis indicated that the sequence was free of such errors (Figure III.10.7). The G+C content of the nucleotide sequence that corresponds to the alpha subunit of the SCS protein-coding region was found to be 72%. No TTA codons were observed in this sequence indicating that this protein was not a direct *bldA* target. Since it was the phosphorylation state of the 32 kDa protein that appeared to be *bldA*-dependent it is likely that this protein represents an indirect *bldA* target and that whatever phosphatase is responsible for dephosphorylation of this protein is the direct *bldA* target.

The *sucD* gene from *Streptomyces coelicolor* was found to be 308 amino acids with a calculated molecular weight of 31,426.84 Daltons and a calculated pI of 6.16. This is in agreement with the total amino acid composition obtained from the University of Victoria Microsequencing Department (Section III.8.3) although the molecular weights differed by ~754 Daltons. Since the gene sequence of *S. coelicolor sucD* was obtained based on homology to the *Mycobacterium sucD*, an alignment was performed

Figure III.10.6. Schematic representation of the DNA in the region around the *sucD* gene on cosmid SC8A6. Direction of gene transcription is shown by the arrows. Unique restriction sites used for cloning are shown with the size of fragment they would generate (see text for details). *SC8A6.05*, gene product is probable TTP-requiring enzyme; *sucC*, gene encoding for a probable succinyl-CoA synthetase beta chain; *sucD*, gene encoding a probable succinyl-CoA synthetase alpha chain; *SC8A6.08*, gene product is probable dehydrogenase; *SC8A6.09*, encodes for a protein with unknown function, tRNA Leu, leucyl tRNA with anticodon GAG; *fusB*, gene encoding possible alternative elongation factor G. Primers used for *S1* nuclease protection analysis and primer extension are indicated by the small arrows.

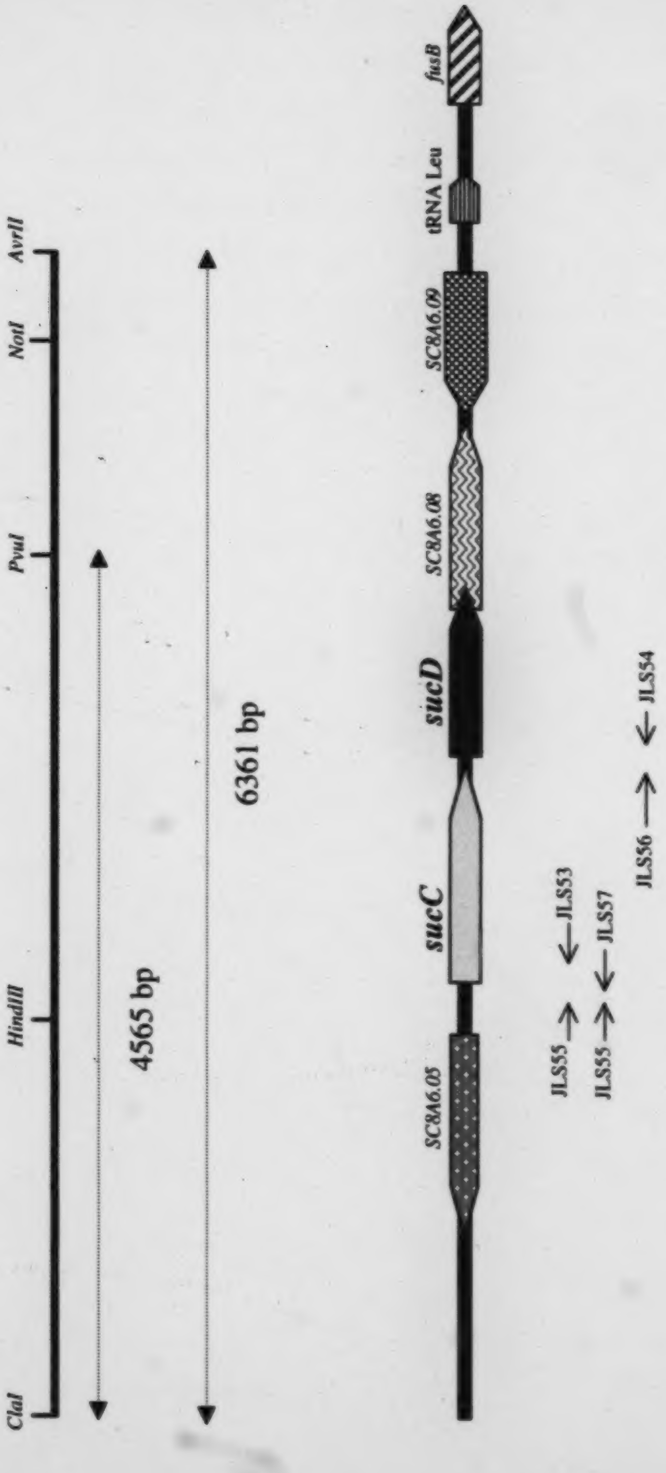
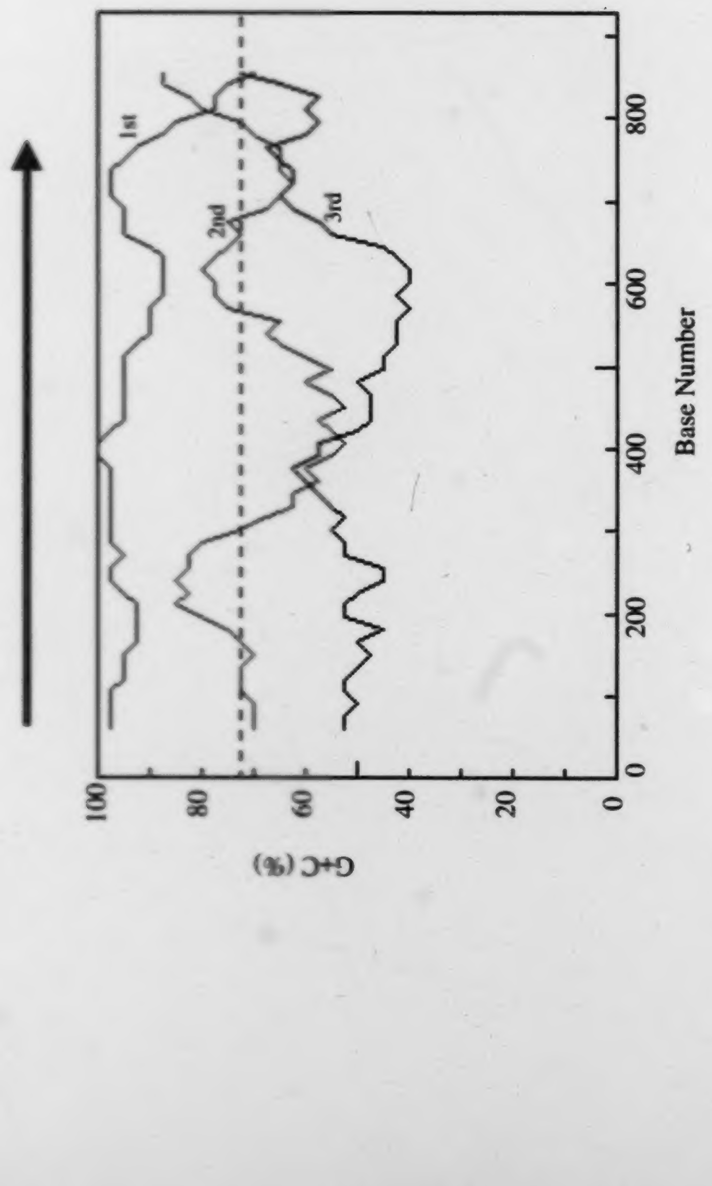

ClaI
HindIII
PvuI
NotI
AvrII
4565 bp
6361 bp
SC8A6.05
sucC
sucD
SC8A6.08
SC8A6.09
tRNA Leu
fusB
JLS55
JLS53
JLS55
JLS57
JLS56
JLS54

Figure III.10.7. Analysis of the *sucD* nucleotide sequence for frame-shift sequencing errors. The complete nucleotide sequence of the *sucD* gene for the alpha subunit of SCS, along with 20 nucleotides upstream and downstream of the protein-coding region was analyzed using a web-based FRAMEPLOT program. The G+C base composition at the first, second and third nucleotides of a window of 40 triplets was scanned and the resulting profile is shown. The arrow indicates the direction of transcription.


G+C (%)
100
80
60
40
20
0
0
200
400
600
800
Base Number
1st
2nd
3rd

for the protein from *S. coelicolor* and *M. tuberculosis* (Figure III.10.8). The gene for the alpha subunit in *M. tuberculosis* is 303 amino acids.

III.11 Generation of a *sucD* Null Mutant Strain

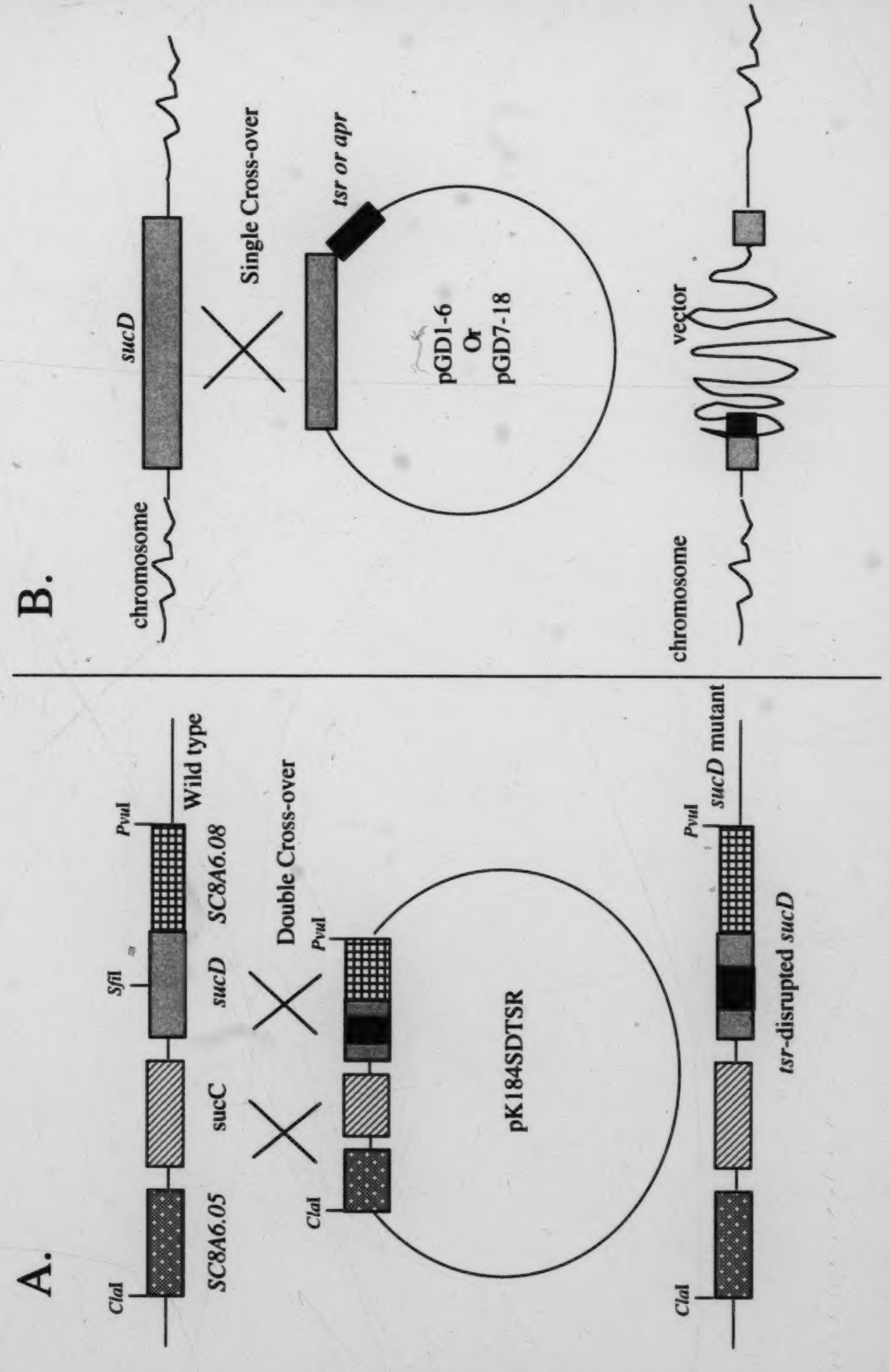
In an attempt to elucidate the role of the succinyl-CoA synthetase in morphological differentiation and antibiotic production, gene disruption experiments were performed. In order to increase the success rate of gene replacement, denatured DNA (Oh and Chater, 1997) was used to transform *S. coelicolor* protoplasts (Hillemann *et al.*, 1991) since this method has been shown to increase the frequency of integrative transformations in *Streptomyces*. The general scheme for generating the recombinant plasmids is shown in Figure III.11.1

A lack of unique restriction sites complicated the design of gene disruption constructs. Attempts were made at constructing a recombinant plasmid that contained the 4565 bp *ClaI/Pvu*I fragment (see Figure III.10.6) which includes DNA upstream of *SC8A6.05* and DNA downstream of the *sucD* gene that encompassed both subunits for the SCS protein. Attempts were made at cloning this 4565 bp fragment into the *Sph*I site of the vectors pK184 and pK194, which differ in the orientation of their MCS (Jobling and Holmes, 1990) and which have the kanamycin resistance marker which makes these vectors selectable in *Streptomyces*. The 4565 bp *ClaI/Pvu*I fragment was only stable in pK184. This construct, designated pJLS4565CP, was sequenced using primers JLS51 and JLS48, which were located at either end of the *sucD* gene. Sequence analysis confirmed that the *sucD* gene was present on the construct. The *tsr* gene was to be cloned into the *Sfi*I site located within the *sucD* gene (Figure III.11.1-A); however, by use

Figure III.10.8. Amino acid sequence alignment of the alpha subunit of succinyl-CoA synthetase of *S. coelicolor* and *Mycobacterium tuberculosis*. Alignment was performed by BEAUTY-enhanced BLAST search (Altschul *et al*., 1990; Worley *et al*., 1995) (http://dot.imgen.bcm.tmc.edu:9331/seq-search/protein-search.html). The amino acid sequences used to design oligonucleotide primers for PCR amplification of *sucD* are underlined. The boxed sequence represents the peptide sequence obtained from microsequencing of the 32 kDa-phosphorylated protein. The consensus sequence is shown. Gaps in sequence are denoted with (-).

```
S. coelicolor:      1   MAIYLTKESKVLVQGMTGAEGMKHTRRMLAAGTDVVGGVNPRKAGRTVDFDNR-----TV 55
                        M+I+L++++KV+VQG+TG+E   HT RML AGT +VGGVN RKAG TV  +++      +
M. tuberculosis:    4   MSIFLSRDNKVIVQGITGSEATVHTARMLRAGTQIVGGVNARKAGTTVTHEDKGGRLIKL 63

S. coelicolor:      56  PVFGSVREGIERTGADVTVVFVPPAFAEAAVVEAADAGVGLAVVITEGIPVHDSVALTAH 115
                        PVFGSV E +E+TGADV+++FVPP FA+ A++EA DA + L VVITEGIPV D+    A+
M. tuberculosis:    64  PVFGSVAEAMEKTGADVSIIFVPPTFAKDAIIEAIDAEIPLLVVITEGIPVQDTAYAWAY 123

S. coelicolor:      116 ARAKG--TRVIGPNCPGLITPGQSNAGIIPPDITKPGRIGLVSKSGTLTYQLMYELRDIG 173
                            G  TR+IGPNCPG+I+PGQS AGI P +IT PG IGLVSKSGTLTYQ+M+ELRD+G
M. tuberculosis:    124 NLEAGHKTRIIGPNCPGIISPGQSLAGITPANITGPGPIGLVSKSGTLTYQMMFELRDLG 183

S. coelicolor:      174 FSTCVGIGGDPVVGTSHIDCLAAFEDDPDTELIVLIGEIGGDAEERAAAHIRAHVTKPVV 233
                        FST +GIGGDPV+GT+HID + AFE DPDT+LIV+IGEIGGDAEERAA  I+ +V+KPVV
M. tuberculosis:    184 FSTAIGIGGDPVIGTTHIDAIEAFERDPDTKLIVMIGEIGGDAEERAADFIKTNVSKPVV 243

S. coelicolor:      234 AYIAGFTAPEGRTMGHAGAIVSGSSGTARAKKEALESVGVRVGSTPTET---ARHVLQAL 290
                         Y+AGFTAPEG+TMGHAGAIVSGSSGTA AK+EALE+ GV+VG TP+ T   AR +L +L
M. tuberculosis:    244 GYVAGFTAPEGKTMGHAGAIVSGSSGTAAAKQEALEAAGVKVGKTPSATAALAREILLSL 303
```

Figure III.11.1. Scheme for generation of recombinant plasmids for gene disruption. (A) Proposed model of integration of *tsr*-disrupted sucD into *S. coelicolor* chromosome at the *sucD* locus by double cross-over. The solid, black box represents the *tsr* gene while the gray box represents the *sucD* gene. The plasmid construct, pK184SDTSR, was to have been derived from the insertion of *tsr* into the *Sfi*I site internal to *sucD* in pJLS4565CP (the 4565 bp *ClaI*/*Pvu*I fragment in pK184). The plasmid pJLS4565CP was found to contain rearrangements upstream of the *sucC* gene. (B) Disruption of the *sucD* gene by single cross-over. A 525 bp PCR-generated fragment internal to the *sucD* gene which possessed *Hind*III and *Xba*I sites, was cloned into the *Hind*III-*Xba*I site of pUC119+Ap^R to generate pGD1-6 and the *Hind*III-*Xba*I site of pAU5 to generate pGD7-18. These recombinant plasmids were used to transform *S. coelicolor* J1501 protoplasts. A single cross-over would result in disruption of the *sucD* gene by vector sequence.


A.
ClaI
SC8A6.05
sucC
SfiI
sucD
SC8A6.08
PvuI
Wild type
Double Cross-over
ClaI
PvuI
pK184SDTSR
ClaI
PvuI
sucD mutant
tsr-disrupted sucD
B.
chromosome
sucD
Single Cross-over
tsr or apr
pGD1-6
Or
pGD7-18
chromosome
vector

of this construct as template for sequencing reactions for S1 nuclease protection assays, it was discovered that the sequence upstream of the *sucC* gene had undergone rearrangement and would not be useful for gene disruption experiments.

An alternative approach to disrupting the sucD gene in wild type *S. coelicolor* was attempted (Figure III.11.1-B). A 527 bp internal fragment of *sucD* was amplified using JLS46 (5'-GGCTAAGCTTGTCACGGTCGTCTTCGTG-3') which possessed a *Hin*dIII site engineered on the 5'-end (underlined), and JLS47 (5'-CCACTCTAGACATCGTCCGGCCCTCG-3') which possessed an *XbaI* site engineered on the 5'-end and denatured *S. coelicolor* J1501 chromosomal DNA as template. An internal fragment was chosen for these experiments because previous attempts at cloning the entire *sucD* gene had shown that such clones were unstable. The amplified product was gel-purified by the method of Zhen and Swank (1993) and cloned into both pUC119 + Ap^R, which contained the apramycin resistance cassette cloned into the *Xba*I and *Eco*RI sites (Dr. S. E. Jensen, personal communication) and pAU5, which possessed *tsr*, cloned into the *Bcl*I site. Both plasmids had been double-digested with *Hin*dIII and *Xba*I for insertion of the *sucD*-containing fragment. Transformants were screened by colony hybridization with the 527 bp amplified product as probe and 2 positives of each construct were sequenced using primers for *sucD* as well as universal primers to confirm they were the correct construct. The pUC119+Ap^R-derived construct was designated pGD1-6 while the pAU5-derived construct was designated pGD7-18 (GD for gene disruption). Due to the restriction-modification system of *S. coelicolor*, the recombinant plasmids pGD1-6 and pGD7-18 were passed through *E. coli* ET12567 before transformation into *S. coelicolor* J1501 protoplasts. The two vectors alone without insert were also passed through *E. coli* ET12567 to serve as a control to ensure that any observed phenotypic change did not arise due to the presence of vector but from the disruption of the gene. Transformation plates were overlaid with either apralan or thiostrepton as described in Materials and Methods (II.9.1). *S. coelicolor* J1501 protoplasts were also plated to assess regeneration frequency so that if no transformants were observed, problems with the protoplast preparations could be ruled out. Some plates of the protoplasts were also overlaid with antibiotic at the same concentration as the gene disruption plates to ensure that no spontaneous resistant colonies were observed. When strains were transformed with vector alone and overlaid with antibiotic, no resistant colonies were observed. In the first attempt at gene disruption, 30 apramycin resistant pGD1-6 transformants were obtained. These colonies were *bald* and produced copious amounts of blue pigment (actinorhodin). The colonies had sunken centres and ridges where the hyphae had fallen over. Five thiostrepton resistant pGD7-18 transformants were obtained. Two of these transformants were bald and produced a blue pigment (actinorhodin) and three were bald, sectored colonies with alternate sections of unpigmented and pink (undecylprodigiosin) pigmented regions. These transformants were subcultured onto Tiger's milk-containing minimal medium supplemented with mannitol and the appropriate antibiotic to maintain selection. All of the colonies were unpigmented on this medium and exhibited sporulation; a phenotype exhibited on this medium by other known *bld* mutants. In an attempt to confirm that this phenotype had resulted from the disruption of *sucD* by vector sequence and was not due to free insert-containing plasmid, it was necessary to isolate total DNA and confirm the gene disruption by Southern analysis. Therefore, sixteen transformants were inoculated into spring bottles containing a 2:3 ratio of Super YEME:Trypticase Soy (TSB) broth with and without antibiotic. Surprisingly, none of the transformants were viable. Some of the known *bld* mutants grow poorly in liquid culture, but are viable under these conditions. Super YEME and TSB were both tried individually as growth media but neither supported growth of the transformants. After several attempts to grow the putative disruptants in liquid culture, it was decided that surface cultures would be used for chromosomal DNA preparations. Eight transformants (four apramycin resistant and four thiostrepton resistant transformants) were grown on cellophane disks on R2YE plates supplemented with Tiger's milk and the appropriate antibiotic. Cultures were harvested and DNA was isolated by Procedure 3 (Hopwood *et al.*, 1985). Southern analysis of *Hin*dIII and *Xba*I digested chromosomal DNA of the eight potential disruptants using random-primer labelled *tsr* (for pGD7-18 transformants) or *apr* (for pGD1-6) as probe did not reveal any hybridization to the antibiotic resistance markers and therefore indicated that disruption of the *sucD* gene had not occurred. A gene disruption experiment with the pGD7-18 construct was repeated. This time R2YE plates supplemented with Tiger's milk were poured to a volume of 25 mL. The transformation plates were overlaid at 16 hours with a final concentration of thiostrepton of 50 μg/mL. This time, only five transformants were obtained; 4 were non-pigmented and had the *bld* phenotype while the other was *bld* and produced a pink (undecylprodigiosin) pigment. When the colonies were subjected to *Streptomyces* colony hybridization and probed with random-primer labelled *tsr*, all five colonies hybridized. The colonies were therefore grown on R2YE + TM plates supplemented with 50 μg/ml thiostrepton, to confirm their ability to grow under thiostrepton selection. After isolation of chromosomal DNA from mycelia scraped from the surface of the agar plates, chromosomal digests of the potential disruptants were performed and subjected to Southern analysis with random-primer labelled *tsr* as probe. Although it cannot be explained in view of the ability to grow on thiostrepton-containing plates, hybridization with the *tsr* probe was not observed. It is possible that since no negative controls were present on the filters for the colony hybridization (because strains lacking a *tsr* containing plasmid are unable to grow in the presence of antibiotic) that the 'hybridization' observed represented binding of the probe to the mycelial debris remaining on the filters. It may not be possible to obtain a null mutant of *sucD*. An alternative approach by which to establish a connection between succinyl-CoA synthetase, antibiotic production and morphological differentiation will have to be investigated in the future.

III.12 Attempted Amplification of the Gene Encoding L2 by Polymerase Chain Reaction

It was determined that the peptide sequence ATVGAVGNAEQSNINWGK obtained from internal peptide sequencing of the 32 kDa phosphorylated protein was the result of a contaminating protein and had homology to the 50S ribosomal protein, L2 of *M. leprae* and *M. bovis* (Figure III.10.3). The molecular weight of the L2 protein from these organisms is ~30 kDa. It is likely that the two proteins could have co-migrated to the same position on the gel and were subsequently excised in the same gel slice.

Since PCR amplification of the *sucD* gene was successful based on primers from the homologous protein in *Mycobacterium spp.*, attempts were made to amplify the gene for the L2 protein by polymerase chain reaction. The primer JLS36 (5'-

ACCCC(GC)TTCGG(TGCA)CT(TC)CC(TGCA)GC-3') and the primer JLS37 (5'-AAGCC(CG)GAGAA(AG)GC(AGCT)(CT)T(AGCT)ATG-3') were designed based on the *M. leprae* protein sequence. Neither standard PCR reaction conditions, nor the Actinomycete PCR protocol yielded any amplification products. Optimization of reaction conditions was not attempted.

The *M. leprae* L2 protein sequence was used to search the Sanger Centre database and the sequence was found to be similar to a probable L2 protein in cosmid StD31. An alignment of the *S. coelicolor* L2 protein from the cosmid sequence with *M. leprae* (Figure III.12.1) and with *M. bovis* (Figure III.12.2) was performed.

III.13 Northern Analysis of *S. coelicolor sucD* Transcripts

If the *sucD* indeed represents the phosphorylated 32 kDa protein, then the enhanced levels of phosphorylation represent a post-transcriptional control of its activity. To rule out any additional regulation at the level of transcription of *sucD*, it was of interest to determine if there was a difference in the amount of transcript observed between the mutant and the wild type strain. To accomplish this, Northern analysis was carried out. RNA was isolated from surface-grown cultures of *S. coelicolor* J1501 (*bldA*$^{+}$) and J1681 (*bldA*$^{-}$) at 15, 18, 24, 36, 42 and 48 hours post inoculation and subjected to Northern analysis. At 15 and 18 hours post-inoculation, the *S. coelicolor* J1501 cultures were in a pre-differentiation state (no antibiotics were produced, nor were aerial mycelia observed). Cultures were fully differentiated by 42 hours post inoculation: they were sporulated and antibiotics were produced. As expected, *S. coelicolor* J1681, a

Figure III.12.1. Amino acid sequence alignment of the 50S ribosomal L2 proteins of *S. coelicolor* and *Mycobacterium leprae*. Alignment was performed by a web-based BEAUTY-enhanced BLAST search (Altschul *et al*., 1990; Worley *et al*., 1995). The amino acid sequences used to design oligonucleotide primers for PCR amplification of the gene encoding the L2 protein are underlined. The boxed sequence represents the peptide sequence obtained from microsequencing of the 32 kDa-phosphorylated protein. The consensus sequence is shown. Gaps in sequence are denoted with (X).

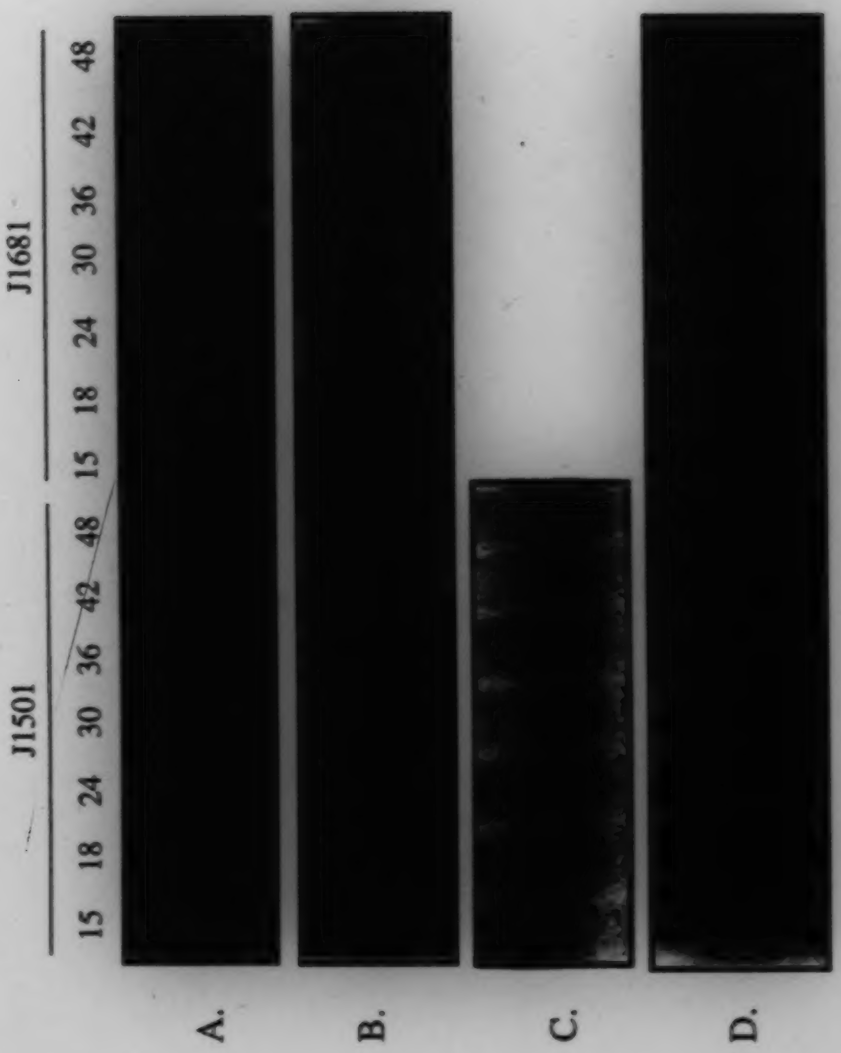
```
S. coelicolor:     1 MGIRKYKPTTPGRRGSSVADFVEVTRSTPEKSLVRPLHSKGGRNNAGRVTVRHQGGGHKR 60
                     M IRKYKPTT GRRG+SV+DF ++TR+ PEK+L+R LH  GGRN  GR+T RH+GGGHKR
M. leprae:         1 MAIRKYKPTTSGRRGASVSDFTDITRTKPEKALMRSLHGHGGRNVHGRITTRHKGGGHKR 60

S. coelicolor:    61 AYRVIDFRRHDKDGVPAKVAHIEYDPNRTARIALLHYADGEKRYILAPRNLQQGDRVENG 120
                     AYR+IDFRR+D DGV AKVAHIEYDPNRTA IALLH+ DG+KRYILAP+ L QGD VE+G
M. leprae:        61 AYRLIDFRRNDTDGVNAKVAHIEYDPNRTANIALLHFLDGKKRYILAPQGLSQGDVVESG 120

S. coelicolor:   121 PGADIKPGNNLALRNIPVGTTIHAIELRPGGGAKFARSAGASVQLLAKEGTMAHLRMPSG 180
                       ADIKPGNNL LRNIP GT IHA+ELRPGGGAK ARSAG+S+QLL KE + A LRMPSG
M. leprae:       121 ANADIKPGNNLPLRNIPAGTLIHAVELRPGGGAKLARSAGSSIQLLGKESSYASLRMPSG 180

S. coelicolor:   181 EIRLVDQRCRATVGEVGNAEQSNINWGKAGRKRWLGVRPTVRGVVMNPVDHPHXXXXXXX 240
                     EIR VD RCRATVGEVGNAEQ+NINWGKAGR RW G RP+VRGVVMNPVDHPH
M. leprae:       181 EIRRVDVRCRATVGEVGNAEQANINWGKAGRMRWKGKRPSVRGVVMNPVDHPHGGGEGKT 240

S. coelicolor:   241 XXXRHPVSPWGKKEGRTRSPKKASNK 266
                        RHPVSPWGK EGRTR P K+SNK
M. leprae:       241 SGGRHPVSPWGKPEGRTRKPNKSSNK 266
```

Figure III.12.2. Amino acid sequence alignment of the 50S ribosomal L2 proteins of *S. coelicolor* and *Mycobacterium bovis*. Alignment was performed by a web-based BEAUTY-enhanced BLAST search (Altschul *et al.*, 1990; Worley *et al.*, 1995). The boxed sequence represents the peptide sequence obtained from microsequencing of the 32 kDa-phosphorylated protein. The consensus sequence is shown. Gaps in sequence are denoted with (X). The amino acid sequences in the region used for PCR primers are underlined.

```
S. coelicolor:     1 MGIRKYKPTTPGRRGSSVADFVEVTRSTPEKSLVRPLHSKGGRNNAGRVTVRHQGGGHKR 60
                     M IRKYKPTTPGRRG+SV+ F E+TRSTPEKSLVRPLH +GGRN  GR+T RH+GGGHKR
M. bovis:          1 MAIRKYKPTTPGRRGASVSYFAEITRSTPEKSLVRPLHGRGGRNAHGRITTRHKGGGHKR 60

S. ceolicolor:    61 AYRVIDFRRHDKDGVPAKVAHIEYDPNRTARIALLHYADGEKRYILAPRNLQQGDRVENG 120
                     AYR+IDFRR+DKDGV  KVAHIEYDPNRTARIALLHY DGEKRYI+AP  L QGD VE+G
M. bovis:         61 AYRMIDFRRNDKDGVNPKVAHIEYDPNRTARIALLHYLDGEKRYIIAPNGLSQGDVVESG 120

S. coelicolor:   121 PGADIKPGNNLALRNIPVGTTIHAIELRPGGGAKFARSAGASVQLLAKEGTMAHLRMPSG 180
                       ADIKPGNNL LRNIP GT IHA+ELRPGGGAK ARSAG+S+QLL KE + A LRMPSG
M. bovis:        121 ANADIKPGNNLPLRNIPAGTLIHAVELRPGGGAKLARSAGSSIQLLGKEASYASLRMPSG 180

S. coelicolor:   181 EIRLVDQRCRATVGEVGNAEQSNINWGKAGRKRWLGVRPTVRGVVMNPVDHPHXXXXXXX 240
                     EIR VD RCRATVGEVGNAEQ+NINWGKAGR RW   RP+VRGVVMNPVDHPH
M. bovis:        181 EIRRVDVRCRATVGEVGNAEQANINWGKAGRMRWKAKRPSVRGVVMNPVDHPHGGGEGKT 240

S. coelicolor:   241 XXXRHPVSPWGKKEGRTRSPKKASNKY 267
                        RHPVSPWGK EGRTR+  K+SNK+
M. bovis:        241 SGGRHPVSPWGKPEGRTRNANKSSNKF 267
```

bldA mutant, did not differentiate. Thirty micrograms of RNA from each time point were denatured with glyoxal and DMSO (Williams and Mason, 1985) and separated on 1.4% agarose gel. Molecular weight markers III and IV (Boehringer Mannheim) were treated in a similar manner and used as size standards. The samples were transferred to a Hybond-N membrane by capillary transfer, UV cross linked and baked in a vacuum oven. The membrane was probed with a random primer labelled 527 bp internal fragment of *sucD* generated by PCR (Plate III.13.1). As a control for RNA loading, the membrane was also probed with a [γ-^{32}P] ATP end-labelled 17-mer, synthetic oligonucleotide BKL54 (5'-CCGCCTTCGCCACCGGT-3') to detect *S. coelicolor* 16S rRNA transcripts. This probe confirmed equivalent loading of the RNA samples on the gel. Two-step RT-PCR was also performed with primers internal to *sucD* (JLS46 and JLS47). Two-step RT-PCR with primers internal to *hrdB* (DBG6 and DBG7) was used as a control to demonstrate even loading among samples. RT-PCR confirmed the results of the northern analysis. In both cases, the transcripts of the *sucD* gene were present in equivalent amounts throughout growth on surface cultures for both the mutant and the wild type strain. Since there was no difference in transcription observed between J1501 and J1681, the differences in phosphorylation that were observed between the two strains must be due to post-translation modification at the level of phosphorylation and not due to increased expression of *sucD* in the mutant strain. The size of the transcript observed by northern analysis was 3.5 kb, agreeing well with the expected size of 3.7 kb if the *sucC*, *sucD*, and overlapping *SC8A6.08* were transcribed as an operon.

Plate III.13.1. Transcriptional analysis of the *sucD* gene. (A) RNA (30 μg) extracted from surface cultures of *S. coelicolor* J1501 (*bldA*$^+$) and J1681 (*bldA*$^-$) at various stages of growth was denatured with glyoxal and DMSO and subjected to electrophoresis on a 1.4% agarose gel. The RNA samples were transferred to a Hybond-N membrane by capillary transfer, UV cross-linked and baked in a vacuum oven. The membrane was probed with a random primer labelled 527 bp internal fragment of *sucD* and exposed to a phosphorimager screen overnight. (B) As a control for RNA loading, [γ-^{32}P] ATP end-labelled BKL54, corresponding to the consensus sequence of *Streptomyces* 16S rRNA sequences, was used to probe the same Northern blot. (C) As a control for RNA loading in two-step RT-PCR reactions, RT-PCR was carried out using 3 μg RNA as template and primers specific for *hrdB* of *S. coelicolor* J1501. Reactions were subjected to electrophoresis on a 5% polyacrylamide gel with a TBE buffer system. The gel was dried under vacuum, wrapped in Saran wrap and exposed to a phosphorimager screen for 5 hours. (D) Two-step RT-PCR of *sucD* with RNA extracted from surface culture time course experiments of J1501 and J1681. Reactions were subjected to electrophoresis on a 5% polyacrylamide gel with a TBE buffer system. The gel was dried under vacuum, wrapped in Saran wrap and exposed to a phosphorimager screen for 5 hours.


J1501
15 18 24 30 36 42 48
J1681
15 18 24 30 36 42 48
A.
B.
C.
D.

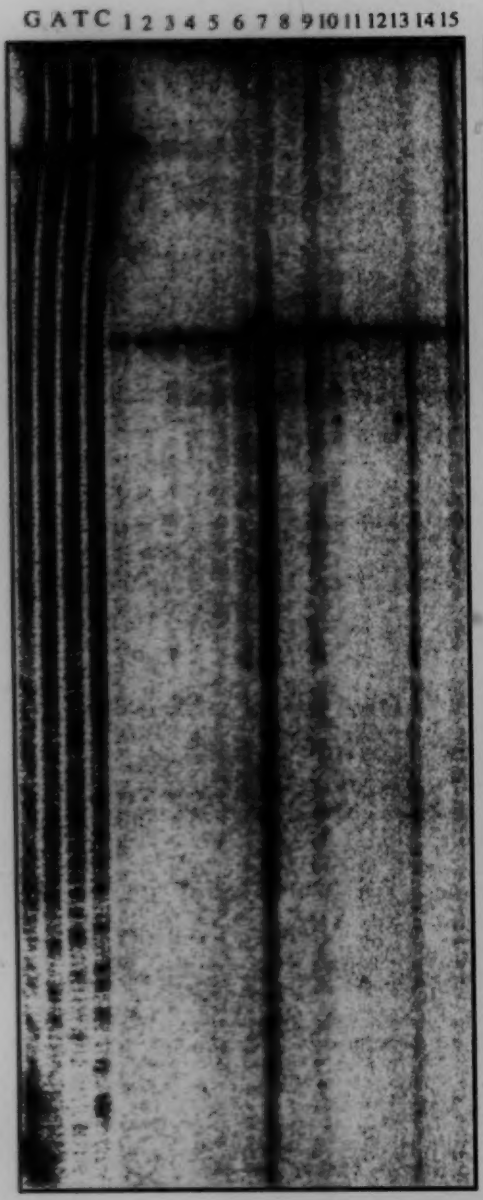
III.14 S1 Nuclease Protection Analysis

In an attempt to identify the promoter and transcription start site of the *S. coelicolor sucD* gene, S1 nuclease protection analysis was performed. The subunits comprising succinyl-CoA synthetase (SCS) in organisms such as *E. coli*, are transcribed as an operon (Buck *et al.*, 1986; Cunningham and Guest, 1998; Spencer and Guest, 1994), therefore it was likely that the same was true for *S. coelicolor*. It was necessary to attempt to map upstream of *sucD* to eliminate the possibility that it had its own promoter as well as to map upstream of *sucC* where the promoter for the operon was expected to be located.

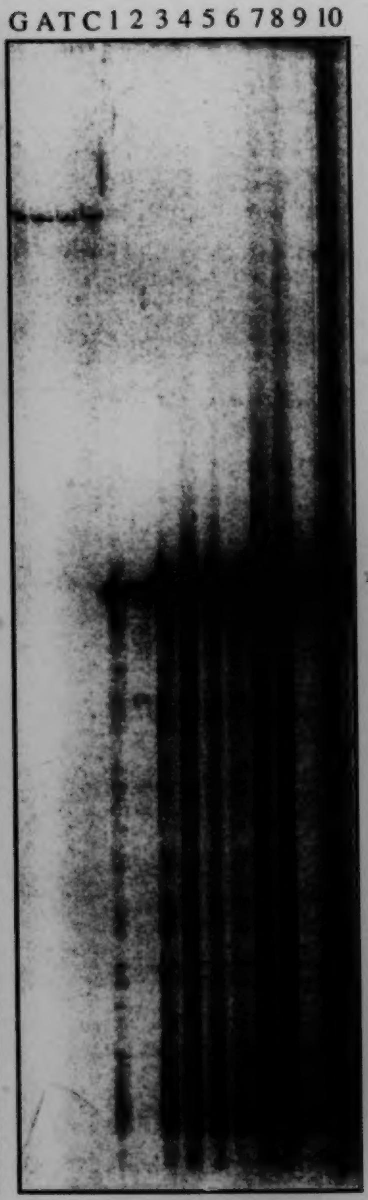
Analysis of the sequence in the *sucC-sucD* intergenic region shows that there are only 14 bp between the two genes, a distance too short to accommodate a promoter. Therefore, if *sucD* is expressed from its own promoter, that promoter would have to be located in the 3' sequence of the *sucC* gene. Therefore, the probe for *S1* nuclease protection of the *sucC-sucD* intergenic region was generated by PCR amplification of a 241 bp fragment using denatured J1501 chromosomal DNA as template. The primers were an 18-mer synthetic oligonucleotide JLS54 (5'-GTGTGCTTCATGCCCTCG-3') corresponding to the sequence 73 bp downstream from the start of the *sucD* coding sequence and a 28-mer synthetic oligonucleotide JLS56 (5'-GCGCAAGCTTCTCACCAAACCGCTCGTC-3') corresponding to a region 185 bases upstream of the translation start of *sucD*. The upstream primer, JLS56, contains a 10 nucleotide non-homologous extension (underlined) at the 5' end which eliminates the need to separate the strands after probe labelling since the 5' labelled, non-homologous end would be removed by S1 nuclease treatment. The use of the non-homologous extension also allows differentiation between full-length protection and probe-probe reannealing since the full-length protected fragments will be 10 nucleotides shorter than the reannealed probe fragments. RNA samples (10-40 μg) isolated from *S. coelicolor* J1501 (wild type) and J1681 (*bldA*-mutant) surface grown cultures were hybridized to the [γ-^{32}P] ATP end-labelled probe and treated with S1 nuclease as described in Materials and Methods (II.13.3). A sample of probe alone was treated with S1 nuclease as a control. Full-length protection of the probe was not observed. It is possible that the transcripts are unstable making it difficult to assay protection of the probe with this region of the mRNA. Since it was likely that the two subunits of the SCS were transcribed as an operon, attention was focused on mapping the promoter upstream of the *sucC* gene.

The probe for *S1* nuclease mapping of the *sucC* gene was generated by PCR amplification of a 301 bp fragment (Probe A) using denatured J1501 chromosomal DNA as template. The primers were an 18-mer synthetic oligonucleotide JLS53 (5'-TCCCGTGTTCCTTGAAGA-3') corresponding to the sequence 48 bp downstream of the start of the *sucC* coding sequence and a 28-mer synthetic oligonucleotide JLS55 (5'-GCGCAAGCTTACGCAGCCGTCGTAGATG-3') corresponding to the region 272 bases upstream of the translation start of the *sucC* coding sequence. The upstream primer contains a 10 nucleotide non-homologous extension (underlined) at its 5' end. The probe was annealed at a temperature 5°C above the estimated Tm of the double-stranded probe, in this case 65.3°C (Plate III.14.1-A). Only probe-probe annealing was observed. The

Plate III.14.1. *S1* nuclease protection analysis of *S. coelicolor sucC* transcripts. (A) Aliquots of RNA extracted from surface grown cultures of *S. coelicolor* J1501 (*bldA*$^+$) and J1681 (*bldA*$^-$) were annealed to 100,000 cpm/2µL Probe A (301 bp PCR generated fragment) at 65.3°C and subjected to *S1* nuclease treatment. Reaction products were separated on a 6% sequencing gel for 2 hours with sequencing reactions generated using pJLS4565CP as template and JLS53 as primer. Gels were fixed in 10% acetic acid: 10% methanol, dried under vacuum and exposed to a phosphorimager screen overnight. Lane: (1) J1501, 15 h; 2) J1501, 18 h; (3) J1501, 24 h; (4) J1501, 30 h; (5) J1501, 36 h; (6) J1501, 42 h; (7) J1501, 48 h; (8) J1681, 15 h; (9) J1681, 18 h; (10) J1681, 24 h; (11) J1681, 30 h; (12) J1681, 36 h; (13) J1681, 42 h; (14) J1681, 48 h; (15) probe alone control. (B) Aliquots of RNA extracted from surface grown cultures of *S. coelicolor* J1501 (*bldA*$^+$) were annealed to either 100,000 cpm/2µL or 200,000 cpm/2µL Probe A (301 bp PCR generated fragment) at 60°C and and 68°C and subjected to *S1* nuclease treatment. Reaction products were separated on a 6% sequencing gel for 5 hours with sequencing reactions generated using pJLS4565CP as template. Gels were fixed in 10% acetic acid:10% methanol, dried under vacuum and exposed to a phosphorimager screen overnight. Reactions in which an annealing temperature of 60°C was used are lane: (1) 24 h, 100,000 cpm/2µL; (2) 42 h, 100,000 cpm/2µL; (3) 24 h, 200,000 cpm/2µL; (4) 42 h, 200,000 cpm/2µL. Reactions in which the annealing temperature was carried out at 68°C are: (5) 24 h, 100,000 cpm/2µL; (6) 42 h, 100,000 cpm/2µL; (7) 24 h, 200,000 cpm/2µL; (8) 42 h, 200,000 cpm/2µL; (9) Probe alone control annealed at 60°C; (10) Probe alone control annealed at 68°C.


G A T C 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

A.


G A T C 1 2 3 4 5 6 7 8 9 10

B.

experiment was attempted again, this time annealing temperatures of 60°C and 68°C were employed (Plate III.14.1-B) but only probe-probe annealing was observed. The sequencing reactions that were run with these experiments used pJLS4565CP as template and JLS53 as primer. This construct contains a 4565 *Cla*I/*Pvu*I fragment which encompasses DNA upstream and downstream of the *sucC* and *sucD* genes and was found to have rearrangements of the DNA upstream of the *sucC* gene, which is why the sequencing reactions did not work. *S1* nuclease mapping was attempted again, this time a newly designed probe was also used. Probe B, which was a 279 bp PCR amplified product using the 18-mer synthetic oligonucleotide JLS57 (5'-CAGGTCCATCTCGGCTCC-3') which spans the start of the *sucC* coding region and the 28-mer synthetic oligonucleotide JLS55. Probe A and Probe B were used with varying amounts of RNA template with an annealing temperature of 55°C. This low annealing temperature was used in an attempt to get more than just probe-probe annealing. Conditions could not be found where a transcription start site could be observed (not shown).

III.15 Primer Extension Analysis

Since S1 nuclease protection analysis failed to identify the transcription start point of the *sucC* operon mRNA, primer extension analysis was performed. The oligonucleotide primer used for primer extension analysis was JLS57 (5'-CAGGTCCATCTCGGCTCC-3'), an 18-mer complementary to the 5' end of the *sucC* coding region. Twenty microgram aliquots of RNA obtained from time course experiments of surface grown *S. coelicolor* J1501 and J1681 were hybridized to [γ-^{32}P] ATP end-labelled

JLS57 at 75°C for 10 minutes and slow cooled to 55°C. Aliquots containing 5 µg of RNA that had been annealed with labelled JLS57 were subjected to reverse transcription and samples containing 2 µg of RNA were loaded onto 6% sequencing gels with sequencing reactions generated by JLS57 (Plate III.15.1) using PCR amplified DNA as template. The 5' end of the mRNA was found to be 49 nt upstream of the start of the *sucC* coding region (Figure III.15.2). Because of the failure of the S1 nuclease protection studies, confirmation that this corresponds to the transcription start site was not obtained. A putative -10 sequence similar to those found in *E. coli*-like *Streptomyces* promoters (Strohl, 1992) was found upstream of the mRNA 5' end. At a spacing of 16 bp, a -35-like sequence was also observed in this region. In order to try to confirm that *sucD* did not have its own promoter, JLS52 (5'-TGGTGAGGTAGATCGCCAT-3') was used for primer extension analysis of mRNA transcripts spanning the 3' end of *sucC* and the intergenic region upstream of the *sucD* translation start. No mRNA 5' ends were observed using this primer. Therefore, it is likely that *sucD* does not possess its own promoter and is transcribed as part of an operon with *sucC* and the downstream dehydrogenase.

Plate III.15.1. Primer extension analysis of *S. coelicolor sucC*. Aliquots of RNA extracted from *S. coelicolor* J1501 (*bldA*$^+$) and J1681 (*bldA*$^-$) grown as surface cultures were annealed to [γ-^{32}P] ATP end-labelled JLS57 and subjected to reverse transcription. Reaction products were separated on 6% sequencing gels with sequencing reactions generated by JLS57 as sequencing primer and PCR amplified product as template. The transcription start point is indicated with (*). The sequence of the complementary strand is shown.

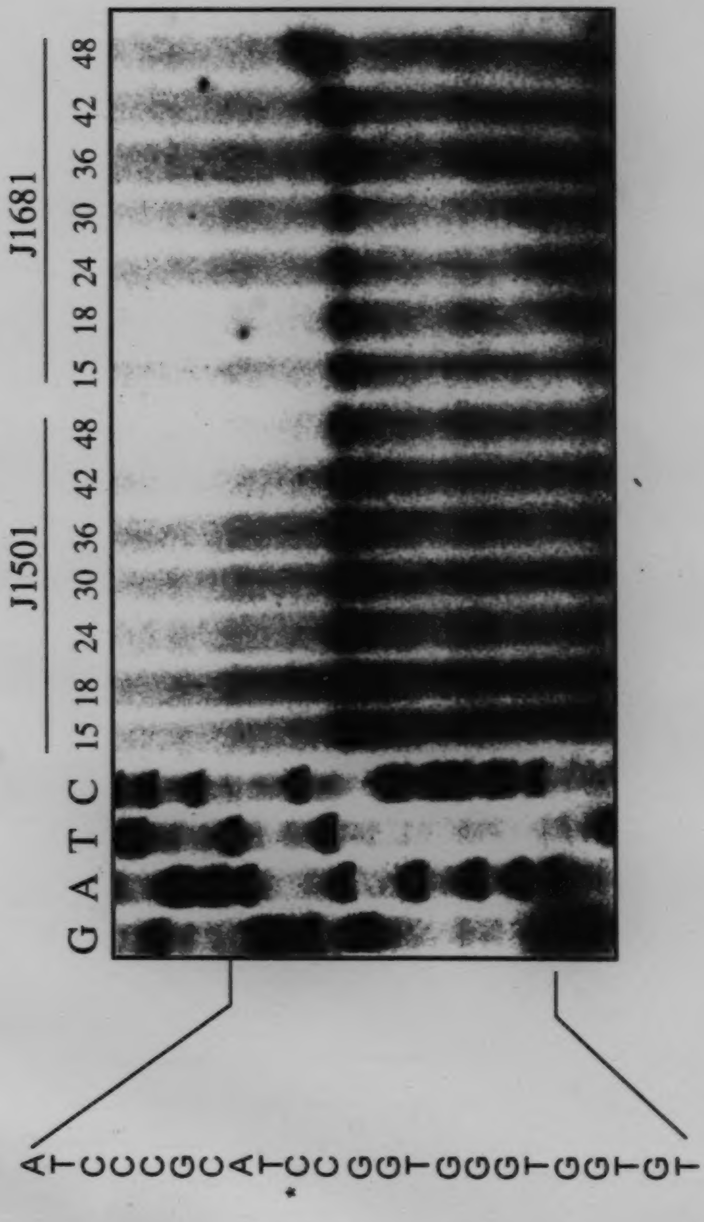

J1681
J1501
48 42 36 30 24 18 15
48 42 36 30 24 18 15
G A T C
ATCCCGCATC*CGGTGGGTGGTGT

Figure III.15.2. Nucleotide sequence and predicted amino acid sequence of the gene encoding the beta subunit (*sucC*) of succinyl-CoA synthetase. The sequence of the intergenic region between SC8A6.05 and the start of the *sucC*-coding region is also shown. The sequence of *sucC* from the translation start site to the termination codon is shown and the predicted amino acid sequence is given directly below. The one letter amino acid code was used. The (*) denotes a stop codon. The numbers at the ends of the lines refer to base pair locations relative to the first base of the translation initiation codon. The transcription start point, as determined by primer extension, is shown with a (•→). Putative -10 and -35 regions of the *sucC* promoter are boxed. The Shine-Dalgarno (S-D) sequence is underlined. A region of ~300 bp upstream and ~300 bp downstream of the transcription start site were manually sequenced, the remainder of the nucleotide sequence comes from the Sanger Centre database for cosmid SC8A6.

```
                                                      GTGCCTTGGCAACGAGGT

      GGCCGCCCGGAGATGACGTCCTGGGTGTCGTCGGGCATGGCGAAGTCCTGTCCCTTCACGA

                                               -35                  -10
      GGGGTTGGGGCGCTCTCGCGGTACATTGCATACAGTCGACGAATACTGTATGAAGCTTGT

               ● •
      TATCCCGCATCCGGTGGGTGGTGTCCAGGGGGCGTGCGGCACTTTCAGTCAGGAGCCGAG
                                                          S-D
   1 - ATGGACCTGTACGAACACCAGGCAAGGGAACTCTTCAAGGAACACGGGATCGTGGTCCCG - 60
     - M  D  L  Y  E  H  Q  A  R  E  L  F  K  E  H  G  I  V  V  P
  61 - AGGGCCGAGGTCACCGACTCGCCCGAACGGGCCCGGGAGATCGCCCGCGCACTCGGCGGA - 120
     - R  A  E  V  T  D  S  P  E  R  A  R  E  I  A  R  A  L  G  G
 121 - CGCGCCGTCGTCAAGGCGCAGGTGAAGACCGGCGGGCGCGGCAAGGCGGGCGGAGTGCGG - 180
     - R  A  V  V  K  A  Q  V  K  T  G  G  R  G  K  A  G  G  V  R
 181 - CTCGCCGCCGACCCGGCCGAGGCCGAGGAGGCGGCGCGGCACATCCTCGGCATGGACATC - 240
     - L  A  A  D  P  A  E  A  E  E  A  A  R  H  I  L  G  M  D  I
 241 - AGGGGACACACGGTCGACACCGTCATGCTGGCCGAACCCTGCGAGATAGAGCGGGAGTTC - 300
     - R  G  H  T  V  D  T  V  M  L  A  E  P  C  E  I  E  R  E  F
 301 - TACGTCTCCTACGTCCTCGACCGGGCGTCCGGCGGCTTCCTCGCCATCGCCTCCGCGGAG - 360
     - Y  V  S  Y  V  L  D  R  A  S  G  G  F  L  A  I  A  S  A  E
 361 - GGCGGCACGGAGATCGAGGAGGTCGCCGCCCGGCGGCCCGAGGCCGTGGCGCGCATCCCC - 420
     - G  G  T  E  I  E  E  V  A  A  R  R  P  E  A  V  A  R  I  P
 421 - GTCGACCCCGCCACGGGCGTGCACACGGCCACCGCGGTACGCATCGCCGACGCCGCCGGC - 480
     - V  D  P  A  T  G  V  H  T  A  T  A  V  R  I  A  D  A  A  G
 481 - CTGCCCCCGCAGACCGTCGACACGCTGGTGCGGCTGTGGAAGGTACTGGTCCGCGAGGAC - 540
     - L  P  P  Q  T  V  D  T  L  V  R  L  W  K  V  L  V  R  E  D
 541 - GCCCTCCTGGTCGAGGTCAACCCGTTGGTCAGGACGGCCGAGGGCCGGATCGTGGCCCTC - 600
     - A  L  L  V  E  V  N  P  L  V  R  T  A  E  G  R  I  V  A  L
 601 - GACGGCAAGGTCACCCTCGACGACAACGCCCGCTTCCGACAGTCCCGTTGGGGCGAGACG - 660
     - D  G  K  V  T  L  D  D  N  A  R  F  R  Q  S  R  W  G  E  T
 661 - AGGCAGGAGGACGCCGACTCCCTGGAGGCGCGGGCCGGCGCCAAGGGCCTCAACTACGTC - 720
     - R  Q  E  D  A  D  S  L  E  A  R  A  G  A  K  G  L  N  Y  V
 721 - AAGCTGGACGGCGAGGTCGGCGTCATCGGCAACGGCGCCGGGCTGGTCATGTCGACACTC - 780
     - K  L  D  G  E  V  G  V  I  G  N  G  A  G  L  V  M  S  T  L
 781 - GACGTGGTCGCCGGCTGCGGTGCCCGCCCCGCCAACTTCCTCGACATCGGCGGGGGCGCC - 840
     - D  V  V  A  G  C  G  A  R  P  A  N  F  L  D  I  G  G  G  A
 841 - TCCGCCCGGGTCATGGCCGACGGACTGTCCGTCGTGCTCTCCGACCCCGACGTGAGGTCC - 900
     - S  A  R  V  M  A  D  G  L  S  V  V  L  S  D  P  D  V  R  S
 901 - GTCCTCGTCAACGTCTTCGGCGGCATCACCGCCTGCGACGCGGTCGCCGACGGCATCGTC - 960
     - V  L  V  N  V  F  G  G  I  T  A  C  D  A  V  A  D  G  I  V
 961 - CGCGCCCTGGACGAGGTCCGGCTCACCAAACCGCTCGTCGTACGCCTCGACGGGAACAAC - 1020
     - R  A  L  D  E  V  R  L  T  K  P  L  V  V  R  L  D  G  N  N
1021 - GCCGCGCGGGGCCGGGCCCTGCTCGACGCCCGCGCGCACCCCCTGGTCGAACAGGCCACC - 1080
     - A  A  R  G  R  A  L  L  D  A  R  A  H  P  L  V  E  Q  A  T
1081 - ACCATGGACGGCGCCGCCCGCCGTGCCGCCCGGCTCGCCACCGCGGCGTCCACCGCCGGA - 1140
     - T  M  D  G  A  A  R  R  A  A  R  L  A  T  A  A  S  T  A  G
1141 - CAGGCCGGATAA - 1152
     - Q  A  G  *
```

IV. DISCUSSION

The primary objective of this project was to identify and clone *bldA* targets, TTA-containing genes that require the *bldA*-encoded tRNA for translation of their mRNAs. Since *bldA* exerts its effect at the translational level (Leskiw *et al.*, 1991a), protein profiles of isogenic *bldA*$^+$ and *bldA*$^-$ strains of *S. coelicolor* were compared by both one- and two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The unlabelled protein extracts were found to have very complex profiles. Since a role for phosphorylation in developmental events had been proposed (Burbulys *et al.*, 1991; Ueda *et al.*, 1993), *in vitro* phosphorylation with $[\gamma\text{-}^{32}\text{P}]$ ATP (Hong *et al.*, 1993; Wang and Koshland, 1981) was used as a means of narrowing the search for phosphorylated *bldA* targets.

In order to maximize the number of phosphorylated *bldA* targets investigated, *in vitro* labelling reactions were optimized by assessing two different *in vitro* labelling methods. It was found that the method of Hong *et al.* (1993) was generally more efficient at labelling proteins from cell-free extracts of *Streptomyces* and was the method of choice. Because some protein kinases require either Mn^{2+} or Ca^{2+} ions for the phosphorylation reaction (Hong *et al.*, 1993), phosphorylation profiles were assessed after the addition of either manganese or calcium. Differences in the phosphorylation status of several proteins were observed when either manganese or calcium was added to the *in vitro* labelling reaction, indicating a requirement for these divalent cations.

The search for phosphorylated *bldA* targets led to the identification of a 32 kDa-phosphorylated protein whose phosphorylation status appeared to be dramatically altered in a *bldA*-deleted strain (Plate III.2.1.1, Plate III.2.1.2, and Plate III.2.3.2). The 32 kDa protein had a consistent level of phosphorylation throughout growth in liquid culture but appeared to be more phosphorylated in the *bldA*⁻ strain compared to the wild type when grown as surface cultures. This result led us to speculate that the phosphorylation state of this protein might be *bldA*-dependent and important for morphogenesis since *S. coelicolor* cannot sporulate in submerged culture. For this reason, the 32 kDa-phosphorylated protein became the main focus of this research project.

Two-dimensional (2-D) gel analysis was undertaken in hopes that this would be an ideal method with which to identify and purify the potential *bldA* target for amino acid sequencing. Despite attempts at optimization, the 2-D gels were irreproducible. One problem encountered was that the protein samples sometimes failed to enter the IEF tube gels resulting in a significant loss of protein. Another limitation of trying to use 2-D gel electrophoresis as a means to identify and purify potential *bldA* targets is that spots which may have a low protein concentration and which overlap with their neighbors are difficult to detect and resolve. Regulatory proteins such as the *bldA* targets, are likely to be present at low levels and may not be detectable by this method. These targets may also not be phosphorylated since not all regulatory proteins are phosphorylated and this analysis would not have addressed those targets. It was observed that there were several phosphorylated proteins that migrated with an apparent molecular weight of 32 kDa when proteins from cell-free extracts were separated by 2-D gel electrophoresis but that they differed in isoelectric point. Efforts aimed at optimization of two-dimensional gels failed to improve the reproducibility observed between gels, therefore, alternative methods for the purification of the 32 kDa protein were investigated.

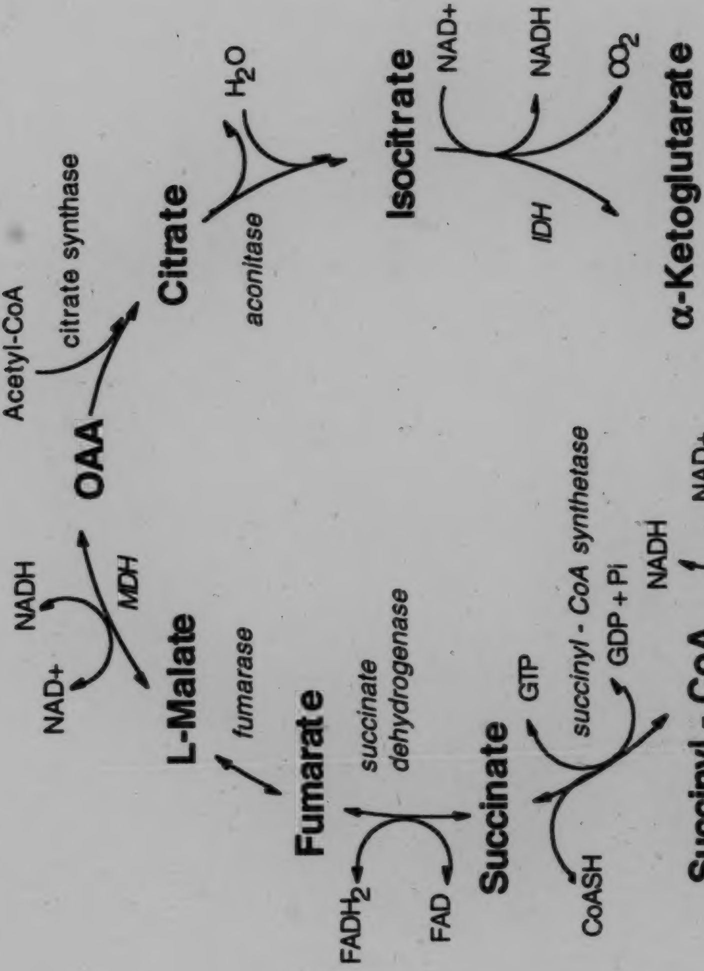
Several purification strategies were utilized in an attempt to purify the 32 kDa phosphorylated protein for characterization. Chromatography of [γ-^{32}P] ATP labelled extracts failed to yield purified protein of the correct size. Even though the labelled cell-free extract was dialyzed to remove unincorporated label, a significant amount of what appeared to be unincorporated label eluted from the column in the first few fractions (Figure III.8.1.5). This seemed to suggest that label was being lost from the labelled proteins during the chromatographic methods employed. If degradation of the protein was responsible for the loss of label, a smear of bands in the SDS-PAGE would have been expected but was not observed. If a small amount of phosphorylated protein had eluted from the column, phosphorylated protein bands should have been observed because of the sensitivity achieved with radiolabelling. Again, this was not observed.

Electroelution of the desired 32 kDa phosphorylated protein from SDS-polyacrylamide gels was also attempted. Although this resulted in the purification of three bands which could be separated electrophoretically, the protein was found to be N-terminally blocked when sent for sequencing to the University of Victoria facility. Unfortunately, whether the N-terminally blocked protein sent for sequence analysis corresponded to the phosphorylated protein of interest could not be verified because the microsequencing facility would not accept radiolabelled samples (at least at the time that we made enquiries). Although radiolabelled samples were run on the same gel simultaneously with unlabelled samples, a final check of the purified protein based on phosphorylation status could not be performed.

The 32 kDa protein was eventually purified by the sequential-gel band excision method (Dr. K.L. Roy, personal communication) and sent for amino acid sequencing.

The protein did turn out to be N-terminally blocked, but a total amino acid analysis of the protein sent for microsequencing at the University of Victoria facility did reveal a protein of 308 amino acids with an estimated molecular weight of 32,181 Daltons. When a similarly purified sample of the 32 kDa phosphorylated protein was sent to the Harvard microsequencing facility, two internal peptide sequences were obtained. Although the two peptide sequences were initially believed to correspond to a single purified protein, they were later determined to be from two distinct co-purifying proteins. The peptide sequence TMGHAGAIVSGSSGTA$\boxed{Q}$AK was determined to be from the alpha (α) subunit of succinyl-CoA synthetase (SCS), an enzyme of the tricarboxylic acid (TCA) cycle (Figure IV.1) encoded by *sucD*. The second peptide sequence obtained was ATVGEVGNAEQSNINWGK and showed homology to the 50S ribosomal L2 protein of *Mycobacterium leprae* and *Mycobacterium bovis*. It is not clear how the protein sample became contaminated with a second protein because all attempts at purification of the 32 kDa phosphorylated protein by the sequential-gel band excision method resulted in what appeared to be a single protein species adequately separated from a second, co-purifying protein. In addition, at least for one of the purified samples, the total amino acid analysis did not suggest the existence of two different proteins. However, had the protein sample sent to the University of Victoria facility contained a contaminating protein, it may have been present in a low concentration, which might have made it undetectable by the method used. On the other hand, the peptide sequencing method employed by the Harvard Microchemistry Facility (MALDI-TOF-MS) was more sensitive; it would therefore detect a low level of contaminating protein that could have resulted from imprecise excision of the band of interest.

Figure IV.1. The tricarboxylic acid (TCA) cycle. Abbreviations: IDH, isocitrate dehydrogenase; α-KGDH, α-ketoglutarate dehydrogenase; SCS, succinyl-CoA synthetase; MDH, malate dehydrogenase.


OAA
Acetyl-CoA
citrate synthase
Citrate
aconitase
H_2O
Isocitrate
NAD+
NADH
CO_2
IDH
α-Ketoglutarate
α -KGDH
NAD+
NADH
Succinyl - CoA
succinyl - CoA synthetase
GDP + Pi
GTP
CoASH
Succinate
succinate dehydrogenase
FAD
$FADH_2$
Fumarate
fumarase
L-Malate
NAD+
NADH
MDH

DNA sequence analysis of PCR amplified *sucD*, revealed that the glutamine (Q; boxed above) identified by peptide sequencing was really an arginine (R) and that a mistake must have been made in the peptide sequence analysis, since that region of the DNA was sequenced four times (twice per strand) and deemed to be correct. The *S. coelicolor* sequence of *sucD* located on cosmid SC8A6 from the Sanger Centre database was a perfect match to the sequence obtained from PCR amplified *sucD* and therefore confirms this result. Sequence analysis of the *sucD* gene did not reveal a TTA codon. This suggests that if the phosphorylation state of this protein is indeed *bldA*-dependent, then this protein is an indirect target. An indirect *bldA* target can be defined as a protein that does not possess a TTA-codon itself but is acted upon by a regulator that does.

The α-subunit of the *S. coelicolor* SCS consists of 308 amino acids and the molecular weight was determined to be 31,426.84 Daltons which agrees well with the apparent M_r of 32 kDa. The calculated pI of the protein product of *sucD* was 6.16.

Since the possibility existed that the 'contaminating' protein represented the 32 kDa phosphorylated protein of interest, the *M. leprae* L2 protein amino acid sequence was used to search the Sanger Centre database. It was found to show homology to the putative L2 protein on cosmid StD31. The *S. coelicolor* 50S ribosomal L2 protein consists of 256 amino acids and has a calculated molecular weight of 28,135.19 Daltons with a theoretical pI of 11.21. The difference in calculated molecular weight between the two copurifying proteins was ~3,292 Daltons. Since this size difference is usually sufficient to be resolved during SDS-PAGE and subsequent Western analysis, the phosphorylated protein observed likely corresponded to the α-subunit of SCS. An alternative possibility is that the L2 protein may have migrated at the same apparent molecular weight as the *sucD* gene product due to its phosphorylation status. Since these two proteins differ significantly in pI, resolution of the two proteins could easily be achieved by two-dimensional SDS-polyacrylamide gel electrophoresis.

At the same time that attempts were being made to purify sufficient amounts of protein for amino acid sequence determination, efforts were made at characterizing the 32 kDa protein. Tyrosine phosphorylation, which has been found to be associated with development in eukaryotes (Fantl *et al.*, 1993; Hunter and Cooper, 1985) was investigated for the 32 kDa protein since the phosphorylation state of the 32 kDa protein appeared to be linked to development. Using one-dimensional SDS-polyacrylamide gel electrophoresis and Western analysis, this protein was found to react with anti-phosphotyrosine antibodies. It was observed that the 32 kDa protein remained phosphorylated after alkali treatment, which should enhance for P-Tyr and P-Thr since P-Ser is labile in alkali (Kamps, 1991), indicating that it may possess P-Tyr or P-Thr. In an effort to verify which amino acids were phosphorylated, phosphoamino acid analysis using two-dimensional thin layer electrophoresis (Boyle *et al.*, 1991) was performed on freshly prepared protein purified by the sequential-gel band excision method. Surprisingly, this analysis demonstrated that P-Tyr, P-Thr, and P-Ser were all present in the sample assayed suggesting that this protein may be subject to many levels of regulation by phosphorylation. There is some evidence to suggest that the *E. coli* α-subunit is also phosphorylated on other residues in addition to the well characterized P-His in the active site (Freestone *et al.*, 1995). The role for the additional levels of phosphorylation of the *E. coli* enzyme has not been investigated.

A limitation of the phosphoamino acid analysis method employed is that it will not identify other phosphorylated amino acids (such as cysteine, lysine, histidine glutamic and aspartic acid (Cooper *et al.*, 1983)) since these amino acids are extremely acid labile (Cooper *et al.*, 1983; Fujitaki and Smith, 1984). This is why P-His could not be identified by the methods used. Identity of the phosphorylation site(s) must be confirmed by additional methods since phosphoamino acid analysis will only identify the *O*-phosphates (serine, threonine, and tyrosine). To accomplish this, site-directed mutagenesis can be used to replace the phosphoacceptor amino acid to a non-phosphorylatable residue (Boyle *et al.*, 1991) to look at the effect on phosphorylation of the protein. For example, alanine can be used to replace serine or threonine and phenylalanine can replace tyrosine. The mutant phenotype can then be assessed and the phosphorylation and phosphoamino acid pattern can be compared in order to establish which residues are phosphorylated. In addition, such mutants could be used to assess whether phosphorylation is required for normal antibiotic production and development.

Since the L2 protein has been shown to be phosphorylated on serine and threonine in *Streptomyces collinus* (Mikulik and Janda, 1997) and because the L2 protein co-purified along with the α-subunit of SCS, the results of the phosphoamino acid analysis do not definitively point to the α-subunit of SCS as being the 32 kDa protein of interest. The fact that P-Tyr was observed could mean that it came from L2, SCS, or a mixture of the two. The phosphorylation profile of L2 has been characterized in a *Streptomyces* strain, yet this analysis failed to reveal a P-Tyr. This makes it seem more likely that the P-Tyr corresponds to the SCS. Furthermore, the addition of Mn^{2+} had no effect on the phosphorylation status of the L2 protein from *S. collinus* (Mikulik *et al.*, 1998) yet was found to enhance phosphorylation of the 32 kDa protein of interest. Before any final conclusions can be made regarding the phosphorylation sites in SCS, in addition to the known P-His in the catalytic site, purified protein must be obtained and the phosphoamino acid analysis repeated. The phosphoamino acid analysis in this work was carried out prior to determination of the identity of the protein and prior to the knowledge that there was a contaminant. As part of planned future work, overexpression of the *sucD* in *E. coli* as a His-tagged fusion will be done to generate antibodies to the α-subunit of SCS. These antibodies will then be used for immunoprecipitation experiments where the immunoprecipitation profiles will be compared with the *in vitro* phosphorylation profiles. Alternatively, if a null mutant of *sucD* can be generated, the phosphorylation profile can be compared with that for J1501 (*bldA*$^+$) and J1681 (*bldA*$^-$), and phosphoamino acid analysis repeated.

The L2 family of ribosomal proteins is highly conserved and is found in both prokaryotes and eukayrotes (Pan and Mason, 1997). L2 is important at the peptidyl transferase center of the large ribosomal subunit (Mikulik and Janda, 1997). Attempts to use PCR to amplify the L2 protein for further characterization were unsuccessful even when the actinomycete PCR protocol was used. In retrospect, with the *S. coelicolor* L2 protein sequence now available for comparison, this may be because the 5' primer was designed based on the amino acid sequence of the *M. leprae* protein (Figure III.12.1). A comparison of the amino acid sequence alignments showed that the *M. bovis* sequence was a 100% match to the *S. coelicolor* L2 amino acid sequence in the area chosen for primer design (Figure III.12.2) while the sequence for *M. leprae* was not. Therefore, it is likely that if primers had been designed based on the *M. bovis* sequence the PCR would have been successful. Unfortunately, when these PCR amplification experiments were initiated, the *S. coelicolor* L2 protein had not been found during a search of the Sanger Centre database and the 50S ribosomal L2 protein was not investigated further.

As would be expected if the phosphorylation state of the 32 kDa protein was *bldA*-dependent, introduction of the *bldA* gene on a high copy number plasmid into the *bldA*$^-$ mutant strain decreased phosphorylation of the 32 kDa protein (Plate III.7.1). Unexpectedly, when the *bldA* gene was introduced on a high copy number plasmid into the wild type strain, the amount of phosphorylation observed for the 32 kDa protein was found to increase (Plate III.7.1) to a level observed for the *bldA*-deleted strain. This may result in the titration of a phosphatase or an intermediate target of the protein due to the presence of *bldA* at high copy in a strain that already possesses normal *bldA* levels. It would be of interest to perform a similar experiment with the *bldA* gene on a low copy number plasmid to investigate this possibility. A few extra copies of *bldA* may not be adequate to titrate the component responsible for dephosphorylation of the 32 kDa protein and therefore the phosphorylation status of this protein in J1501 harboring the *bldA* gene on a low copy number plasmid should be informative.

The *sucD* gene encoding the α-subunit of succinyl-CoA synthetase (SCS) from *S. coelicolor* is located downstream from the gene encoding the β-subunit, *sucC*. Interestingly, the *sucD* gene overlaps a putative dehydrogenase at its 3' end (Figure III.10.6). The function of this dehydrogenase in relation to the TCA cycle and the impact it may have on differentiation or antibiotic production remains to be elucidated since the putative dehydrogenase does not show homology to known dehydrogenases normally associated with SCS or the TCA cycle. The expression of succinyl-CoA synthetase in *E.*

coli is coordinately regulated with α-ketoglutarate dehydrogenase (Buck *et al.*, 1986), an enzyme which does not share homology with the dehydrogenase encoded downstream of *sucD* in *S. coelicolor*. Northern analysis provided evidence that *sucC, sucD* and the putative dehydrogenase genes are all transcribed as an operon in *S. coelicolor*. The analysis also indicated that regulation of *sucD* (and presumably the rest of the operon) does not occur at the transcriptional level since no temporal differences were observed at different time points during growth, and since there were no differences between the wild type and the *bldA* mutant. This confirmed that the enhanced level of phosphorylation observed for the 32 kDa protein represented a post-transcriptional control mechanism (Plate III.13.1).

SCS has not been extensively studied in Gram positive bacteria. Given the role of other TCA cycle enzymes in sporulation in *Bacillus* including aconitase (Alen and Sonenshein, 1999; Craig *et al.*, 1997; Dingman *et al.*, 1987; Fortnagel and Freese, 1968; Hanson *et al.*, 1964), isocitrate dehydrogenase (Fortnagel and Freese, 1968; Hanson *et al.*, 1964; Jin *et al.*, 1997; Rutberg and Hoch, 1970), α-ketoglutarate dehydrogenase (Fortnagel and Freese, 1968; Rutberg and Hoch, 1970), succinate dehydrogenase (Fortnagel and Freese, 1968; Friden *et al.*, 1987) and malate dehydrogenase (Warren, 1968), it seems reasonable to suggest that SCS could play a significant role in the sporulation of *Streptomyces*. In *B. subtilis*, the levels of enzymes of the TCA cycle peak when batch cultures enter stationary growth phase (Fortnagel and Freese, 1968; Hanson *et al.*, 1963; Ohne, 1975) and are coincident with the onset of sporulation. Mutants of *B. subtilis* which lack TCA cycle enzymes, exhibit a significant decrease in ATP after glucose is exhausted from the medium and are unable to sporulate (Carls and Hanson, 1971). Recently, an aconitase gene has been disrupted in *Streptomyces viridochromogenes* and was found to impair both morphological and biochemical differentiation (Schwartz *et al.*, 1999). So far this is the only example of a disrupted TCA cycle enzyme in *Streptomyces*. As with the *S. viridochromogenes* aconitase, gene replacement experiments were attempted in order to elucidate the role that SCS (also known as succinate thiokinase) plays in morphogenesis and antibiotic production in *S. coelicolor*. However, as described above, all attempts at generating a null mutant failed. Interestingly, mutants have been detected in *B. subtilis* which are defective in each step of the TCA cycle except for the reaction catalyzed by SCS (Carls and Hanson, 1971; Fortnagel and Freese, 1968; Freese and Fortnagel, 1969; Hoch and Coukoulis, 1978; Magnusson *et al.*, 1983). In *B. subtilis*, an intact TCA cycle is not required for vegetative growth; therefore, it would seem to suggest that generation of a null mutant of SCS should be possible. Perhaps the inability to generate a null mutant suggests an additional role for this protein, a possibility that could involve the other phosphorylation sites putatively identified on both the *E. coli* and *S. coelicolor* SCS. Since a null mutant could not be generated to assess the phenotypic effect SCS has on morphological differentiation and antibiotic production in *S. coelicolor*, it would be informative to perform site-directed mutagenesis on the phosphorylated active site histidine by replacing it with an unphosphorylatable amino acid. In *E. coli* the conserved histidine in the phosphorylation site of the α-subunit of SCS has been replaced by asparagine (Mann *et al.*, 1991) and by aspartic acid (Majumdar *et al.*, 1991). Both of these amino acid substitutions resulted in complete loss of enzymatic activity. If such a mutant form of SCS can be generated in *S. coelicolor*, it should be able to confirm the *bldA*-dependence of the phosphorylation status of this protein and how morphogenesis and biochemical differentiation are affected by the phosphorylation state of this protein, since it should enable the distinction between its catalytic activity (by removing the P-His from the catalytic site) and any putative regulatory role other phosphorylation sites might impose.

Cell differentiation and growth processes are a reflection of complex, tightly regulated pathways. Changes in energy metabolism play a role in determining the potential for growth and development (Birney *et al.*, 1996). Succinyl-CoA synthetase is the only enzyme in the citric acid cycle that catalyzes a substrate-level phosphorylation reaction of GDP or ADP. This reaction is completely reversible (with a ΔG° of -0.7 kcal mol^{-1}) and allows for the production of nucleoside triphosphate (NTP) during aerobic metabolism and the synthesis of succinyl-CoA for anabolic reactions (Birney *et al.*, 1996). Succinyl-CoA synthetase catalyzes the interconversion of succinyl-CoA and succinate accompanied by the production or hydrolysis of GTP (Kornberg, 1966; Nishimura and Grinnel, 1972).

All Succinyl-CoA synthetases characterized thus far possess α and β types of subunits. In Gram negative bacteria, such as *E. coli*, the enzyme is a tetramer of two α and β subunits ($\alpha_2\beta_2$; α = 29.6 kDa; β = 41.4 kDa) (Bridger, 1984; Wolodko *et al.*, 1986). The subunits in eukaryotic SCS are larger (α = 32.1 kDa and β = 42.5 kDa) (Wolodko *et al.*, 1986). The α- and β-subunits of *S. coelicolor* SCS are closer in size to the eukaryotic subunits than to the subunits from *E. coli*. In *E. coli*, the specific histidine residue which is phosphorylated-during catalysis is His-246 in the α-subunit (Buck and Guest, 1989;

Wang *et al.*, 1972). The *S. coelicolor* α-subunit does possess the highly conserved histidine residue that is phosphorylated as part of the catalytic cycle of this enzyme (Bridger, 1971) (Figure III.10.5), however, as discussed above, the limitation of the phosphoamino acid analysis procedure employed prevented confirmation that this residue is phosphorylated on the purified 32 kDa protein. There are two known forms of this enzyme: A-form and G-form. In the case of the *E. coli* enzyme, adenine nucleotides are preferentially used in the substrate level phosphorylation reaction and this form is referred to as the A-form of the enzyme. G-form enzymes are predominantly found in eukaryotes and Gram positive bacteria and appear to use guanine nucleotides exclusively (Birney *et al.*, 1996; Weitzman *et al.*, 1986). The G-form enzymes function as dimers composed of a single α and a single β subunit. At present, it is unknown which form of the enzyme exists in *S. coelicolor*, since this was not investigated in the present study.

Freestone *et al.* (1995) showed that the α-subunit of the *E. coli* SCS was phosphorylated with either ATP or GTP as phosphate donor and that Mn^{2+} enhanced phosphorylation of this protein. Furthermore, the phosphorylation of this protein was stable under alkaline conditions but not completely stable to acid hydrolysis (Freestone *et al.*, 1995) suggesting the possibility of P-Tyr and P-Thr. There are no reports in the literature which provide a precedent for phosphorylation of tyrosine on this enzyme. It will therefore be important to confirm the phosphoamino acid analysis of the SCS from *S. coelicolor*. If it turns out that the SCS is indeed tyrosine phosphorylated it would mean another level of regulation is involved with this TCA cycle enzyme and could help identify a mechanism for its possible involvement in regulation of antibiotic production and morphological differentiation. So far, the only evidence for such an involvement is the *bldA* dependence of its phosphorylation level.

Succinyl-CoA synthetase has not generally been considered to be a critical control point for the flow of substrates into the TCA cycle of *E. coli* (Birney *et al.*, 1996). NDP and NTP have been shown to share a common binding site on the enzyme (Cha and Parks, 1964). Nucleoside diphosphate kinase (NDK) has been shown to interact with a GTP-binding protein and succinyl-CoA synthetase and is hypothesized to regulate SCS in *E. coli* and *Pseudomonas aeruginosa* (Kavanaugh-Black *et al.*, 1994; Kimura and Shimada, 1990; Nickerson and Wells, 1984).

Birney *et al.* (1996) demonstrated that GDP altered the phosphorylation of the *E. coli* SCS by enhancing the level of the phosphoenzyme formed when ATP is used as phosphate donor. This group went on to suggest that carbohydrate metabolism can be uncoupled from energy metabolism at the level of SCS when GDP concentrations are low due to an as yet unidentified effector believed to be a guanine nucleotide binding protein. When GDP concentrations are high, the effector-mediated inhibition of SCS phosphorylation by ATP is relieved. Although it is unknown if the same holds true for G-form enzymes (and *S. coelicolor* SCS is closer in size to eukaryotic SCS and may therefore be a G-form enzyme), this may partially explain the results obtained when *bldA* was introduced into a *bldA*$^-$ strain on a high copy number plasmid. Recall that as expected if the phosphorylation status of the 32 kDa protein was *bldA*-dependent, introduction of *bldA* into the mutant resulted in a decrease in the phosphorylation state of this protein. It is possible that GDP concentrations are high in the *bldA*-deleted strain and therefore a *bldA*-dependent effector is either inactive or absent. If this were true, with introduction of *bldA* at a high copy number, the *bldA*-dependent effector would either be produced or simply activated and a decrease in the phosphorylation status of the protein would be observed indicating a low GDP concentration. The fact that introduction of the *bldA* gene in a wild type strain resulted in an increase in the apparent phosphorylation of the SCS α-subunit seems to indicate that there is another level of *bldA*-dependent regulation at play. It could be that there is a critical concentration of GDP required in the cell and that production or activation of inhibitor beyond a certain level results in its inactivation, leading to the observed increase in SCS phosphorylation.

The TCA cycle has three functions: supplying biosynthetic precursors (such as α-ketoglutarate, succinyl-Coenzyme A, and oxaloacetate), generating energy, and creating reducing power (Nakano *et al.*, 1998). A disruption of the TCA cycle would cause a defiency in the precursors required for secondary metabolism. The TCA cycle provides precursors to 10 of the 20 amino acids used for proteins: six are synthesized from oxaloacetate and four from α-ketoglutarate. The catabolism of valine, leucine and isoleucine plays a pivotal role in supplying fatty acid precursors for macrolide and polyether antibiotic formation under certain growth conditions (Craster *et al.*, 1999; Denoya *et al.*, 1995; Tang *et al.*, 1994; Zhang *et al.*, 1999). Succinyl-CoA is a precursor for tetrapyrolles which are prosthetic groups in cytochromes. Therefore, amino acid pools would be affected if the flow of carbon through the TCA cycle was stalled or defective at the level of SCS. Precursors for secondary metabolism such as those required for polyketide biosynthesis, which requires the branched chain amino acids, would then be affected. In this way, primary metabolism and antibiotic production are intimately linked.

In *Streptomyces,* it has been documented that depending on the availability of nutrients, a significant fraction of pyruvate and α-ketoglutarate, for example, are excreted into the medium (Dekleva and Strohl, 1987; Karandikar *et al.*, 1997; Madden *et al.*, 1996). As available supplies of the preferred catabolic substrates such as glucose are exhausted, the excreted acids are transported back into the cell with a concomitant increase in medium pH. These acids are then metabolized *via* the TCA cycle. Süsstrunk and others (Liu *et al.*, 1985; Redshaw *et al.*, 1976; Surowitz and Pfister, 1985; Süsstrunk *et al.*, 1998) observed that *bld* mutants irreversibly acidified their medium and that pyruvate and α-ketoglutarate were the organic acids in the medium. If the TCA cycle was defective at SCS, α-ketoglutarate concentration might not be sensed and perhaps not imported for utilization. Under these conditions the medium would remain acidified and as seen in the work by Süsstrunk *et al.* (1998) no aerial mycelium would be formed. The step that links metabolism of carbon and nitrogen is the conversion of α-ketoglutarate to glutamate and glutamine (Fisher, 1992). In Gram negative organisms, the expression of nitrogen metabolism genes is determined by the ratio of the intracellular concentrations of α-ketoglutarate, which is a reflection of carbon source availability, and glutamine, a reflection of nitrogen availability (Fisher, 1992). In *Streptomyces*, the excretion of acid is dependent on the availability of a readily utilizable carbon source. Ammonium ions suppress acid over-production while acid is overproduced in the presence of nitrate and amino acids (Madden *et al.*, 1996). Levels of pyruvate and α-ketoglutarate vary depending on the nitrogen source, being high with nitrate, alanine, aspartate and glutamate, and low with glycine and lysine (Madden *et al.*, 1996). It is conceivable that a sensor which senses the pH of the extracellular environment or a response regulator responsible for transducing the signal may be defective or absent in a *bldA* mutant. Furthermore, this signal may be transduced to SCS *via* the putative P-Tyr, P-Ser, and P-Thr residues where they may exert a regulatory influence.

It is known that *S. coelicolor* produces γ-butyrolactones (Horinouchi, 1993). A-factor, a γ-butyrolactone, is synthesized from one glycerol derivative and one β-keto acid. The β-keto acid is synthesized from three acetate and one isobutyrate in the same way as the elongation of carbons in polyketide biosynthesis (Horinouchi, 1993; Horinouchi, 1996). If precursors are not being generated because of a deficient TCA cycle then γ-butyrolactone production and polyketide biosynthesis will both be influenced. In this way, the synthesis of an autoregulator may be intimately linked with polyketide biosynthesis. If catabolic precursors are limited, the organism may be unable to produce a γ-butyrolactone which acts as a signal for morphological differentiation and antibiotic production. Another way that non-catalytic SCS could influence morphological differentiation and antibiotic production is at the level of the GTP, cAMP and ppGpp pools. For example, ATP pools would be expected to decline significantly if substrate level phosphorylation were inhibited due to an incomplete TCA cycle, as observed in *B. subtilis* TCA cycle mutants (Carls and Hanson, 1971). If the substrate level phosphorylation reaction was inhibited at the level of SCS, there would likely be less ATP in the cell for sporulation-related processes. The GTP pool would ultimately be affected, and this would in turn indirectly affect cAMP pools and also likely ppGpp pools as well.

Study of the SCS may provide insight into the proposed link between the switch in the metabolism of organic acids and the onset of antibiotic production and morphological differentiation. The possibility that the SCS is phosphorylated on serine, threonine and tyrosine, in addition to the histidine at the catalytic site, seems to suggest that SCS plays a regulatory role in addition to its catalytic role. Since many *bld* mutants, as well as the *cyaA* mutants which exhibit a bald phenotype, show a decrease in the pH of the culture medium (Süsstrunk *et al.*, 1998), it has been suggested that the onset of antibiotic production and differentiation involves a shift in metabolism to the utilization of the organic acids in the medium. It would be of interest to test *sucD* or other SCS mutants for irreversible acidification of the medium. If a connection can be established between SCS and the shift in metabolism this would enhance our understanding of the interplay between primary and secondary metabolism and the signals for morphological differentiation.

In addition to the points discussed above, other evidence suggests that antibiotic production can be triggered by an imbalance in metabolism. Undecylprodigiosin is derived partly from proline (Gerber *et al.*, 1978). Since proline biosynthesis appears to be constitutive in *S. coelicolor*, it has been suggested that undecylprodigiosin may serve as a sink for excess proline (Hood *et al.*, 1992). The need to remove surplus proline might reflect the role that it plays as an osmoregulant in other bacteria (Killham and Firestone, 1984). If the TCA cycle was not completing a full round of the cycle, then proline levels could be indirectly affected. When proline levels are low, it will be used for growth and survival processes and not incorporated into undecylprodigiosin.

As has been outlined above, control of morphological and chemical differentiation involves multiple regulatory processes. Some regulatory events are dedicated to either morphogenesis or to antibiotic production, while some are shared between the two pathways. The regulatory connection between metabolism and antibiotic production is obviously intimately associated. Studies such as the one described here will hopefully enable the elucidation of the signals that initiate the differentiation process in *S. coelicolor.*

Future Directions

In vitro protein phosphorylation experiments have shown that there are several proteins that are temporally phosphorylated and several that show differences between *bldA*$^+$ and *bldA*$^-$ strains. These proteins may also represent regulators of antibiotic biosynthesis and are potential *bldA* targets. These proteins should be purified, cloned and characterized.

Since the *S. coelicolor* genome sequence has been almost completely determined, all of the direct *bldA* targets will soon be known. Already several potential *bldA* targets with unknown function have been identified. It is now important to begin to investigate their functions by mutagenesis and gene disruption to assess their effect on antibiotic production and morphological differentiation. It is of interest to determine which proteins these potential targets interact with so that more pieces can be added to the regulatory puzzle governing differentiation in *Streptomyces.*

To date, primary metabolism has been understudied in the streptomycetes. Since the precursors for secondary metabolism are generated by primary metabolism, shared regulatory components need to be investigated. To this end, investigation of where *bldA* targets fit into the regulatory scheme controlling the switch from primary to secondary metabolism will be important for elucidating the regulatory processes.

The regulatory mechanisms which underlie the physiological and environmental signals responsible for the morphological and biochemical differentiation observed in *Streptomyces* species are only starting to be deciphered. Until the targets of the regulators, such as those encoded by the *bld* genes, are identified, the full implications of the multi-layered regulation exhibited by *Streptomyces* will remain elusive.

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